University of Évora

ARCHMAT
(ERASMUS MUNDUS MASTER IN ARCHaeological MATerials Science)

Mestrado em Arqueologia e Ambiente (Erasmus Mundus –ARCHMAT)

Analysis of organic residues and lead content in Roman amphorae from Southwest Lusitania

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List of abbreviations

Abbreviation	Explanation
AMDIS	Automated Mass spectral Deconvolution and Identification System
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
DAG	Diacylglycerol
DHA	Dehydroabietic acid
EI	Electron Ionization
FT-IR	Fourier Transform Infrared spectroscopy
GC-MS	Gas Chromatography coupled with Mass Spectrometry
HCl	Hydrochloric acid
KOH	Potassium hydroxide
LA-ICP-MS	Laser Ablation Inductively Coupled with Plasma Mass Spectrometry
MAG	Monoacylglycerol
MDHA	Methyl-dehydroabietic acid
NIST	National Institute of Standards and Technology
Pb	Plumbum
TAG	Triacylglycerol
TLE	Total Lipid Extract
TMS	Trimethylsilyl
TRA	Time Resolved Analysis

Abstract

The villa of São Cucufate (Vidigueira, southern Alentejo, Portugal) was an agricultural center with Roman occupation from the 1st century AD. For the analysis of organic residues and lead (Pb) content in amphorae sherd from this archaeological site, Gas chromatography coupled with mass spectrometry (GC–MS) and Laser ablation inductively coupled with plasma mass spectrometry (LA-ICP-MS) were applied. GC-MS enabled to identify long chained alkanes, palmitic, stearic, pimaric, isopimaric and abietic acids. The presence of diterpenoids yielded evidence that the amphorae were waterproofed with resinous substances for carrying liquid or semi liquid products. Unfortunately, the absence of phenolic acids in wine biomarker extraction could not help to correlate between the samples with resinous substances and lead content. Moreover, the presence of a wide range of organic compounds is a clear identification of use and reuses of amphorae possibly for plant products, vegetable oils, and animal products during its lifetime.

Resumo

'Análise de resíduos orgânicos e teor de chumbo em ânforas romanas do sudoeste da Lusitânia'

A vila de São Cucufate (Vidigueira, Alentejo, Portugal) foi um centro agrícola com ocupação Romana desde o século I. Para a análise de resíduos orgânicos e teor em chumbo (Pb) em fragmentos de cerâmica provenientes deste sítio arqueológico, foram utilizadas as técnicas de cromatografia gasosa acoplada a espectrometria de massa (GC–MS) e espectrometria de massa com fonte indutiva de plasma com amostragem por ablação laser (LA-ICP-MS). A análise por GC-MS permitiu a identificação de alcanos de cadeia longa, ácidos gordos palmítico e esteárico, e ácidos pimárico, isopimárico e abiético. A presença de diterpenóides permite colocar a hipótese de que as ânforas terão sido impermeabilizadas com substâncias resinosas, para permitir o transporte de materiais líquidos ou semi-líquidos. Infelizmente, a ausência de ácidos fenólicos após extracção dos biomarcadores vínicos não permitiu fazer a correlação entre conteúdo resinoso, vinho e presença de chumbo. Adicionalmente, a presença de uma vasta gama de compostos orgânicos é um sinal de reutilização das ânforas possivelmente para produtos derivados de plantas, óleos vegetais, e produtos animais durante o tempo da sua utilização.

CHAPTER 1: INTRODUCTION

1.1 Archaeological context

The Roman ruins of São Cucufate (which is also known as the Roman ruins of the villa of São Cucufate, Ruins of Santiago, Archaeological ruins of São Cucufate or Roman villa of São Áulica) is a Romanesque archaeological site. It is located on the ruins of a Roman-era agricultural farm in the civil parish of villa of Frades, in the municipality of Vidigueira, southern Alentejo, Portugal. It is considered as one of the most significant archaeological sites in Southwest Lusitania for revealing different aspects of Roman occupation in ancient times.

Figure 1 The Roman ruins of São Cucufate (photograph by Carole Raddato, distributed under a CC-BY 2.0 license)

Chronologically, it was found that there were several stages of human occupation in different periods in this archaeological site. Geographically easily accessible landscape, naturally fertile soil and enough sources of water made it possible for choosing it as an occupational site for growing agricultural demand and socio-economic development. The beginnings of the convent were laid down in the 1st century with the construction a small Roman villa. It followed the model of architectural design in that period built around the baths and peristyle (Mareco, 2007). There are three phases of construction in São Cucufate. The first villa, built in the middle of 1st century AD, was demolished to build a second villa between the 3rd and 4th centuries. The massive complex that is visible today dates from the middle of 4th century. The

villa was abandoned in the mid-5th century or possible earlier at the end of the 4th century (Mareco, 2007). From this period, traces of a garden still remain together with a stone tank. Inside the building, there are vaulted rooms that would have been used to store wine and olive oil containers, agricultural products of the region, and very much appreciated by the Romans.

Figure 2 Location of the Roman ruins of São Cucufate in the map of Portugal (created using ArcGIS® software by Esri, http://www.arcgis.com/home/webmap/viewer.html?useExisting=1)

Around the 9th century, a convent was established on the ruins of the Roman villa, which persisted until the late 12th century AD. In 1254, the ecclesiastical parish of São Cucufate was installed in the convent, under the supervision of the monastery of São Vicente de Fora. The Augustine canons that lived in the convent were later followed by Benedictine monks. Around the 17th century, the buildings were abandoned by the monastic community, although one hermit monk remained. With a few discontinuities, transformations and adaptations, the occupation of this space extended until the 18th century, primarily since the contiguous area could be utilized for its rich soils and abundance of water, to establish a small garden and residence. The chapel continued to serve the small local community probably until the 18th century (Mendonça, 1993).

The Roman villa of S. Cucufate, classified as a National Monument since 1947 and excavated between 1977 and 1983 by the Portuguese-French team directed respectively by Jorge de Alarcão from the University of Coimbra and Robert Étienne from the University of Bordeaux, constitutes one of the most important archaeological and architectural monuments of the region, in which three overlapped Roman villa witness three successive epochs of rural life (Étienne et al., 2016).The archaeological finds collected during the 1970's and 1980's campaigns include osteological remains and a wide pottery collection, which includes common ceramics, amphorae and other containers.

1.2 Structure of the study

This paper comprises of five chapters with different perspective of research methodologies, acquired information, research aims-objectives and result-discussion.

Chapter one starts with the introduction and includes some other basic beginning parts of the research. This chapter includes a brief explanation of the research background with the literature review in previous years at the same or other related fields of study. It's one of the key chapters to get an idea about all other research works related to this study and importance of this research work for future archaeometric approaches on this field of studies. It also provides rationale for the selection of the research area. Moreover, the first chapter contains explanation of the research aim and objectives and explains research structure.

Chapter two focuses on the working principles of the analytical techniques used in this study. It begins with the discussion on GC-MS analytical technique for organic residue analysis and wine biomarker extraction; and then LA-ICP-MS analytical technique with the applicability for lead content analysis is also explained in this chapter.

Chapter three is all about the materials and methods. It focuses on the sampling methods, extraction of organic compounds from amphorae sherds, total lipid extraction, wine biomarker extraction, Gas Chromatography Mass Spectrometry (GC-MS) analysis and Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysis.

Chapter four is one of the most important sections of this paper focusing on the results of applied methodologies and findings. This part discusses the interpretation of the result of organic residues in amphorae by GC/MS, analysis of total lipid extract, analysis of wine biomarker and analysis of lead profile in amphorae by LA-ICP-MS.

Chapter five which is the last chapter of this paper focuses on the conclusion of this study. It comprises many discussions on implications of research findings, practical recommendations for action, research contribution, recommendations for future research and finally the limitations of the study.

1.3 Research Background

Analysis of organic residues and lead content in Roman amphorae from Southwest Lusitania demanded a broader background research for developing more reliable and acceptable methodological approaches on this field of study. Necessarily, all the potential literary sources on the related field of study were observed and followed for the accuracy. Here in the section of this paper some of the previously published important work and its influence on this study are discussed briefly.

The first important study of amphora was performed in 1899 by Heinrich Dressel, who recorded examples of painted inscriptions and shapes in amphorae. He was a German archaeologist, who developed a typology for classifying ancient amphorae. He was able to separate 27 kinds of different typological orientation and all the other shapes came later on and were not catalogued by Dressel. The names of these shapes relate either to their origin, to the discoverer's name, or to the site of discovery (Dressel, 1899).

Callender was also one of the pioneers in studying amphorae. He described amphorae simply as a container and stresses the differences between amphorae and the other classes of pottery. Pottery, by his opinion, were manufactured and sold as objects to be used in their own right, while amphorae were simply carriers of their contents: "it was after all the latter (i.e. the particular the commodities that they were destined to carry) which was being sold and not the amphora" (Callender, 1950).

'Natural resins of art and archaeology their sources, chemistry, and identification' by John S. Mills and Raymond White (Mills, 1977) is considered as one of the pioneering research articles for the methodological approaches in the identification of resins in organic residue analysis. The authors in this study tried to conduct a survey from their time to previous 15 years of scientific research on resins chemistry and tried to recombine it from scattered field of chemical, phytochemical and technological literature. They presented some successful identification examples of resins through the application of GC.

'Gas Chromatographic Identification of a Resinous Deposit from a 6th Century Storage Jar and Its Possible Identification' by Myra Shackley was another significant work in this field of research. In this study, the author conducted research on the contents of a storage jar considered as Byzantine origin from the castellum of En Boqeq (Israel) with the help of GC. It was possible to identify highly oxidized pine resin by this analytical technique. It was very likely that this resinous substance was being used for

waterproofing, caulking and to make a soft greasy or viscous substance used as ointment or for lubrication (unguent) (Shackley, 1982).

'Pine wood origin for pitch from the Mary Rose' by the authors R.P. Evershed, K. Jerman and G. Eglinton in the year 1985 was another earlier approach in this particular field of study. Through that studies they had found out the evidence that pitches from Mary Rose were from pine wood (Evershed et al., 1985).

'The Survival of Food Residues: New Methods of Analysis, Interpretation and Application' by the authors R.P. Evershed, C. Heron, S. Charter in the year 1991 yielded better understanding of the nature of organic matter of archaeological interest. Chemists were faced with the task of characterizing a wide range of natural products. Gas chromatography (GC) and combined GC/mass spectrometry (GC/MS) were the methods of choice for the analysis of complex lipid mixtures. Application of these techniques to the investigation of amorphous residues preserved in potsherds would appear to hold considerable promise for use in studies of diet and vessel function (Evershed et al., 1991).

In the work of Regert prehistoric glues were analysed. In this article, the triterpenoids and diterpenoids related to the historical periods. It was shown that during prehistoric times, people mostly used birch bark tars. During Bronze Age, pine resin began to be utilized in a big amount. It was also shown that diterpenoids like dehydroabietic, abietic and 7-oxodehydroabietic acids are markers of pine resins (Regert, 2004). In Colombini's studies on pottery vessels from Roman times (Colombini et al., 2005) it was also established that the presence of characteristic diterpenic biomarkers enables to assess the use of both pine resin and pine pitch.

One of the most recent studies was performed by J. Font et al. (2007). In this work, the resinous materials from the interior surfaces of two Roman and one Iberian amphora were studied with Fourier transform infrared (FT-IR) spectroscopy. Gas chromatography-mass spectrometry (GC-MS) was also used in order to compare the results with those of FTIR. The diterpenic nature of samples from amphorae was established (Font et al., 2007).

'The study of pitch via gas chromatography mass spectrometry and Fourier-transformed infrared spectroscopy: the case of the Roman amphoras from Monte Poro, Calabria (Italy)' by Francesca Izzo et al. (2013). In this paper the resinous materials from the interior surfaces of Roman amphorae and the contents of two vessels (called Kadoi) coming from Monte Poro, in Calabria (Italy) were studied. The organic materials were identified by Fourier-transformed infrared spectroscopy (FT-IR) and gas chromatography coupled with mass spectrometry (GC-MS). The presence of monocarboxylic acids and

terpenic species shows that the organic residues were of vegetable origin or mainly consist of vegetable-based resins. Moreover, the presence of characteristic diterpenic biomarkers permits to recognize the use of pine resin and pine pitch, while the presence of methyl dehydro-abietic acid is likely linked to the use of wood tar and not only to the pine pitch (Izzo et al., 2013).

'Identifying wine markers in ceramics and plasters using gas chromatography mass spectrometry. Experimental and archaeological materials' by Pecci et al. (2013) is another potential source of literature followed for wine biomarker extraction methodology (Pecci et al., 2013).

'Prehistoric wine-making at Dikili Tash (Northern Greece): Integrating residue analysis and archaeobotany' by Nicolas Garnier introduced a new two-step analytical protocol that allowed the reliable structural identification of red wine thanks to the presence of dark grape (tartaric, malic and syringic acids) and fermentation markers (succinic and pyruvic acids) in a smashed, large, coarse jar and a jug excavated inside a Neolithic house destroyed by fire around 4300 BCE at the site of Dikili Tash in northern Greece (Garnier & Valamoti, 2016).

'Use and reuse of amphorae. Wine residues in Dressel 2–4 amphorae from Oplontis Villa B (Torre Annunziata, Italy)' by A. Pecci et al. (2017) carried out organic residue analyses on thirteen samples from eleven Dressel 2–4 amphorae recovered at Villa B at Oplontis (Torre Annunziata, Southern Italy) in order to identify their content and to characterize their visible lining. Although the content of Dressel 2–4 amphorae is usually thought to be wine, no residue analyses have been carried out until now to verify it. Analyses were carried out with gas chromatography coupled to mass spectrometry (Pecci et al., 2017).

1.3.1 A brief introduction to Roman amphorae

During the period of Greek and Roman times, a typical pottery container, known as amphorae, was developed with the purpose of storage and transport. These pottery containers are considered a potential source of archaeological research as amphorae were used for large scale movement of solid, liquid and semi-liquid foodstuffs during its lifetime of uses and re-uses. It is obvious that, unlike other pottery containers, amphorae fit a wide range of sizes and shapes. Many amphorae were large, two-handled, spiked or with a rounded bottom. But of course, there are exceptions, making 'amphora' not a typological category, but instead it's a functional group (Peacock & Williams, 1986). From different research on the petrology of amphora fabrics, it was demonstrated that some classes were produced simultaneously in

many regions, and some exported types became widely copied. The complete identification of a vessel takes account of both fabric and form (Blakely, 2014).

There is no unified typological series for all amphorae shapes, and a combination of Dressel's 1899 typology, vessels from site-based typologies (Haltern, Camulodunum, Carthage etc.) and the typologies developed for amphoras from particular sources (e.g. Beltrán for Spanish amphoras, Gauloise for Gaulish, the Africana or Tripolitana series for North African material) is used nowadays. Also, the same type or form of amphorae can be termed with different designations depending on the authors choices, geographical diversity and languages (Peacock & Williams, 1986).

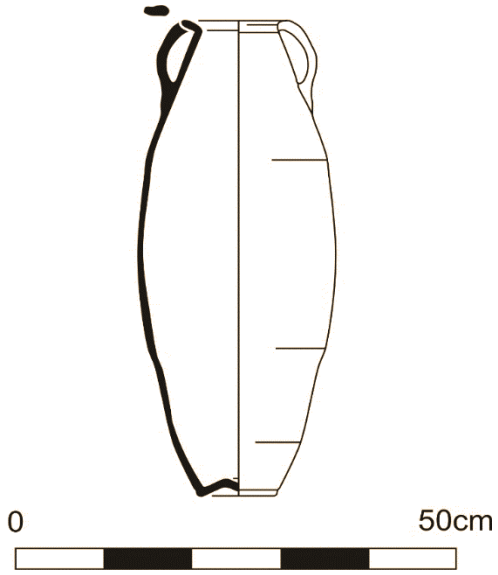


Figure 3 Drawing of Lusitana 9/ Sado2 type amphorae at 1:10 scale (retrieved from http://archaeologydataservice.ac.uk, courtesy of Carlos Fabião, taken by Penny Copeland)

In this research the amphorae from the Roman villa of São Cucufate are termed as Lusitana 9 / Sado 2, a typology firstly described by Diogo Dias (1990; 1991), who studied examples from the Sado valley, but also identified this typology at the kiln centers of Tejo valley, such as Porto dos Cacos and Quinta do Rouxinol. Mayet and Da Silva (1998) have classified this as the Sado 2 type in their publication of the Pinheiro kilns (Mayet & Silva, 1998).

These types of amphorae are the most unusual shape container compared with other containers described and classified on the Lusitania production centre, and with very little information. It's a fusiform vessel, with a big opening, almost without neck and the handles curving sweep away from the body and neck. It has another main characteristic, the flat bottom with a ringed base. There are few chronological proposals introduced with the indication of gaps between the 4th and 5th century, whereas author Diogo Dias (1987)

admitted that the production began before 3rd century (Fabião, 1998). The similarity of the thin wall, top, handle and bottom among the fragments, made it confuse to distinguish Lusitana 9 from Lusitana 3 type. As to the content of this amphora, considering its wide mouth almost without neck, apparently inappropriate to the transport of liquids, and considering the production context, it was suggested that these amphorae could have been used for carrying fish products (fish sauce or salted fish) (Etienne & Mayet, 2000). Diogo Dias (1990; 1991), Mayet & Da Silva (1998) also supported the same hypothesis of carrying fish products within these amphorae. But the author Carlos Fabião, on the year 1998, in his paper titled as 'O vinho na Lusitânia: reflexões em torno de um problema arqueológico' argued that these amphorae could have been used for transporting wine. He mentioned that if the wine content of Lusitana 3 type is accepted, then it is not illogical to argue that the same centre of production might have produced other containers for carrying wine (Fabião, 1998).

Table 1 Distinctive characteristics for the amphoras of Lusitana 9/ Sado 2
(Source: http://archaeologydataservice.ac.uk/archives/view/amphora_ahrb_2005/character.cfm?id=356)

Characteristics	**Type**	**Short description**
Rim	Everted	The type of rim which becomes gently wider towards the top.
Shoulder	None/smooth	There is no distinguishable shoulder. The body of the amphora is uninterrupted.
Handles in profile	Bowed	The handles form a broad, curving sweep away from the body and neck of the amphora. They are generally longer than the 'curved' handles.
Handles in section	Ovoid/ elliptical	The handle appears to be ovoid or elliptical in section.
Neck	None	There is no distinct neck. The body of the amphora progresses smoothly upwards to the rim.
Body	Ovoid	The body is ovoid or elliptical in profile.
Base	Ringed	The base contains a foot ring.
Capacity	None	Not specified.
Height	Generalized	50-60cm
Rim diameter	Generalized	13-15cm
Fabric	Hard-fired, gritty reddish fabric	Normally coarse fabric containing frequent grains of quartz, long strands of mica and a scatter of discrete grains of feldspar (plagioclase, microcline and potash).

1.3.2 Generalized concept of contents in amphorae

The contents of amphorae can be a very vital source of information for revealing the ancient diet, agriculture, socio-economic structure, trade routes and provenance. A long list of contents within a wide range of commodities has been discovered by previous studies, among those it is obvious that the bulk movement of wine and olive-oil is responsible for most amphorae recovered from archaeological sites (Tyers, 1996). In addition to the wine and olive oil, other contents were stored in amphorae, such as different types of fish products, fruits, grains, grape juices, nuts and olives. In the ancient times, Greeks, Romans and other Mediterranean people of antiquity were familiarized with this type of transport of commodities, as nowadays we are transporting liquids, semi-liquids and perishables goods sealed in bottles or cans, varying in size and shape (Evershed, 2008).

1.3.2.1 Nature of organic residues

Organic residues are mainly referred to the amorphous materials discovered in archaeological sites and are considered as a potential source of information for archaeologists and archaeological scientists. They're composed of organic compounds from the remains of organisms such as plants and animals and their waste products in the environment. Basic structures are created from cellulose, tannin, cutin and lignin, along with other various proteins, lipids, nucleic acids, amino acids and carbohydrates. Being amorphous in nature, organic residues show complexity for its proper identification which demands chemical analysis for more defined and sophisticated traceability. The advances in sophisticated archaeometric analytical techniques combined with chemical and biochemical approaches has tremendously improved the scope of organic residue analysis and enabled detection at molecular level. Visible and absorbed organic residues are found in association with pottery containers and serve as initial means of classification (Heron et al., 1993).

Visible residues are referred to the organic residues which usually survive either on the inner or outer parts of the vessel and exhibit its presence by a patina, deposits or encrustations. The visible residues on the outer part of the vessel are mainly flaky substances consisting largely of amorphous carbon resulting from heating over fire during the its lifetime and the interior surface mainly exhibits the paste, layer, charring of food or other organic materials. It was a common trend in Roman and Greek times to waterproof the ceramic vessels with resinous substances for reducing the permeability of porous fabrics,

during the period of manufacturing or for the purposes of use and reuse (Rice, 1987). The permeability of porous fabrics in ceramics is a phenomenon primarily responsible for the absorption of organic residues. Absorbed residues can be both food and nonfood residues, which are usually not visible with unaided eyes (Heron et al., 1993).

From the very beginning, pottery vessels have been used for food storage, food preparation and consumption of meals. yielding plenty biomarkers as evidence of the vessels' content. It is obvious that lipids of different origin can be traced for the purposes of storage or transport of oils. Also, different unsaturated fatty acids can arise from the contact with plant or vegetable remains during its lifetime. A wide range of nonfood residues can be identified in ceramic vessels, depending on degradation, hydrophobicity and leaching. Nonfood residues are mainly used for the proper manufacturing of ceramic vessel, protecting its permeability and increasing high temperature tolerance (Rice, 1987).

1.3.3 Analysis of organic residues from archaeological pottery and the archaeological biomarker concept

Organic residue analysis has a great potential in answering archaeological questions regarding diet and subsistence practices, as well as the ancient trade of goods and raw materials, technology (including vessel production, use and provenance), resource acquisition/exploitation, and the domestication of plants and animals. It should be emphasized that the greatest potential for organic residue analysis on ceramics is from its application at the site level. Such data can then contribute to the interpretations regarding the archaeological questions discussed above, for example, dietary reconstruction, technology and resource acquisition, all on local, national and global scales (Historic England, 2017).

For the analysis of organic residues, developments in analytical chemistry are one of the main driving forces, associated with the advancement of chromatographic and mass spectrometric instrumentation. In terms of resolving or detecting biomolecular components from minimum amounts of preserved organic residues, modern analytical methods have proved its effectiveness. Archaeological biomarker concept is one of the best approaches to analyze the organic residues from ancient ceramics. For the better understanding of past human activity, archaeological biomarker concept incorporates different analytical approaches which can pave the way to biomolecular analysis of archaeological remains. It's a method being developed based on matching chemical structures and their distribution from the archaeological samples with the presence of chemicals in the organisms known to have been exploited in the past

(Manhita et al., 2014). Macro remains from pottery sherds are easily associated with the contamination from handling or soil remains. Instead, unglazed ceramic vessels contain and preserve the chemical fingerprint of the organic residues which were in contact with the object during its lifetime of uses and reuses (Roffet-Salque et al., 2017).

Organic residue analysis of archaeological pottery has successfully addressed few important questions with the most relevant answers. Previously these questions were mainly answered with many uncertainties with the hypothetical knowledge. The type of commodities which were processed or stored in archaeological vessels are now detectable through the application of organic residue analysis. Terrestrial animal fats are very complicated to trace with a high level of accuracy, but the application of isotopic approaches paved the way to distinguish between ruminant and non-ruminant fats from the dairy products. Aquatic fat products (fishes and marine mammals) are possible to be detected by organic residue analysis. Plant remains such as resins, tars, bitumen, oils, waxes and beeswaxes can also be identified, although some compounds are very degradable due to their unsaturated chemical structure (Manhita et al., 2014). Harsh methods (acidic extraction) in organic residue analysis can help to recover the biomarkers of these plant products (Alessandra Pecci et al., 2013).

Examples of chemical structures of lipids found in absorbed and amorphous organic residues are given below.

Animal and plant fats

Identification of archaeologically degraded animal and plant fat source can be determined by stable carbon isotope composition of palmitic (C_{16}) and stearic (C_{18}) acid (Manhita et al., 2014). Plant lipids markers can be identified by the presence of alkanols, alkanoic acids, alkanes and ketones. Sterols can also be a potential biomarker for plant and animal lipid materials, usually cholesterol is the most abundant animal sterol, whereas sitosterol and campesterol are most abundant in plants.

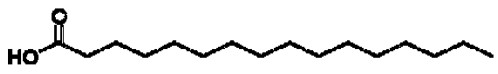


Figure 5 Palmitic acid

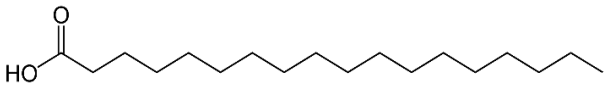


Figure 4 Stearic acid

Aquatic products

Biomarkers of aquatic products can be mainly determined by the presence of long-chain (C20 and longer) unsaturated acids, alkanoic acids, isoprenoid fatty acids and vicinal dihydroxy acids (Heron et al., 2010).

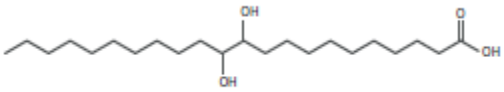


Figure 6 Vicinal dihydroxy acid 11,12-dihydroxydocosanoic acid

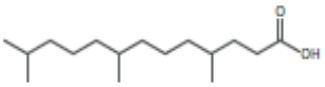


Figure 7 Isoprenoid fatty acid (4.8,12-TMTD)

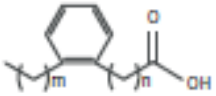


Figure 8 ω-(o-alkylphenyl) alkanoic acid

Resins, tars and pitches

The diterpenoids pimaric acid, isopimaric acid, abietic acid and dehydroabietic acid can be considered potential biomarkers of resins, tars and pitches in organic residue analysis (Jerković et al., 2011). Moronic acid, oleanoic acid, masticadienoic acid and isomarticadienoic acid also can be identified.

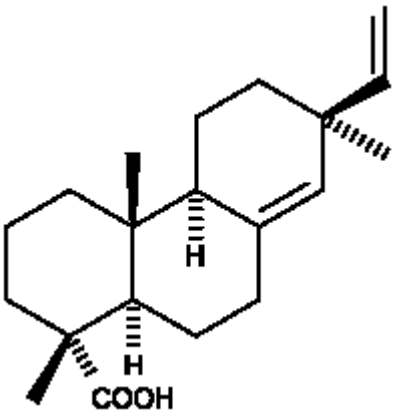


Figure 10 Pimaric acid

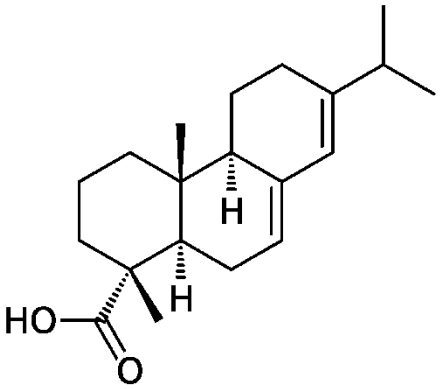


Figure 9 Abietic acid

Beeswax

The major components for beeswax are long-chain odd-carbon-numbered n-alkanes, long-chain alcohols, even-numbered free fatty acids and alcoholic esters of fatty acids (C40-C52). Other compounds such as sterols, terpenoids and saccharides may also occur (Rieley et al., 1991; Logan, 1995). Lignoceric acid and the compound shown in Figure 11 is commonly found in beeswaxes.

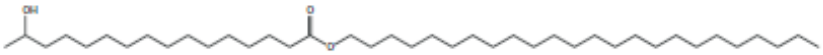


Figure 11 Hydroxy wax ester (tetracosanyl 15-hydroxypalmitate)

Plant leaf waxes

Plant leaf waxes can be identified by different kinds of wax ester, n-alkanols, even numbered long chain fatty acids and long chain n-alkanes.

Figure 12 n-alkane

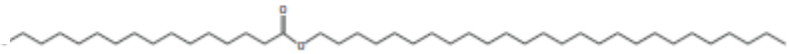


Figure 13 Wax ester

The process of extraction of absorbed residues encompasses modern contamination due to sample handling and storage (skin lipids and cholesterol). Lately developed sampling protocols involve mechanical removal of contaminated surface associated with the soils of the potsherds using drilling tools. This sampling protocol was found appropriate for both museum collections and newly excavated ceramic shreds and did not require much amount of sample extraction, 2-3 grams of clean potsherds yielded enough lipids for analysis. Traditional lipid extraction is accomplished using an organic solvent mixture, like chloroform:methanol (Roffet-Salque et al., 2017).

1.3.4 Lead and the Romans

Lead poisoning occurs when this heavy metal is ingested by organisms willingly or reluctantly. Lead is known as *plumbum* (Pb) since ancient times and in modern time lead poisoning is also known as plumbism (Reddy & Braun, 2010). From different literary sources we came to know that renal failure (kidney disease), birth defects, gout and mental disabilities were common among Roman adults. Different historians claimed that overwhelming consumption of lead-containing sweeteners and wines was one of the reasons for gradual decline of Roman empire. Lead poisoning was also responsible for disorders in reproductive system in roman times (Reddy & Braun, 2010).

Lacking a source of sugar such as sugar beets or sugar cane, Romans made use of lead-rich sweeteners, prepared by boiling down grape juice, must, into sapa, defrutum, or carenum, each with varying degrees of water content to yield different degrees of sweetness. Roman sapa worked effectively as a preservative because of lead's inhibitory effects on enzyme systems in living organisms; this inhibition has a lethal effect on bacteria and thus prevented spoiling of the wines to which sapa was added (Nriagu, 1983).

Analysis of lead in Roman skeletons from Herculaneum, an ancient Roman town destroyed by a volcano, found that 6 of 55 skeletons contained bone lead concentrations between 100-200 ppm, compared to 20-50 ppm in modern American bones. These skeletons were thought to be those of persons suffering from lead poisoning, although the social status of those persons is unknown. Unfortunately, few Roman skeletons are available for this type of analysis, especially for population analysis, because the Romans cremated bodies of the deceased, especially during the first two centuries of the Roman Empire (Reddy & Braun, 2010).

Considering the present study, if the samples (Lusitania 9 amphora sherds from São Cucufate) were in contact with this type of lead-enriched wine, we should see lead enrichment in depth profile of samples by analysis using LA-ICP-MS: the inside part of the amphora should be more enriched in lead than the outside.

1.4 Aims and objectives

For this research, sherds of Lusitana 9 / Sado 2 amphorae were selected for laboratorial analyses. In previous research works, while some authors defended that these amphorae were used for transport of fish products, other authors defended that it might have been used to transport wine. An article published by Carlos Fabião in the year 1998 entitled as ‘O vinho na Lusitânia: reflexões em torno de um problema arqueológico’ is one of the pioneering works. Previous authors like Diogo Dias (1990; 1991) and Mayet and Da Silva (1998) associated the Lusitana 9 / Sado 2 typology with the purposes of carrying fish products (possibly salted fish or fish sauce) based on the amphorae production centres near to river Tejo and Sado. However, Carlos Fabião argued that this type could have been used for transporting wine (Fabião, 1998). This hypothesis will be investigated in this research. Focus will be given to these amphorae contents, and to decide if they were used to carry wine or not by applying wine biomarker extraction protocol, GC-MS and LA-ICP-MS.

Resins (*Pinaceae* sp.) were widely used as adhesives or gluing materials in Roman times for waterproofing ceramics. In previous works, resins were found as a universal material being used in Roman ceramics (Font et al., 2007). GC/MS analysis of the extracts from amphorae sherds will yield the chemical composition of the organic residues in the amphorae sherds, including resinous materials. According to some ancient authors, Romans boiled grape must in lead vessels to concentrate sugars and allow lead to sweeten the wine, whereas lead worked as a potential compound for the long-term preservation of the wine. For investigating that hypothesis, focuses will be given on experimental procedures to detect lead in the amphorae. If the samples were in contact with this type of lead-enriched wine, a lead enrichment will be observed in depth profile of samples using LA-ICP-MS: the inside part of the amphora should be more enriched in lead than the outside.

CHAPTER 2: WORKING PRINCIPLES OF APPLIED ANALYTICAL TECHNIQUES

2.1 GC-MS analytical technique for organic residue analysis

Organic residue analysis is a complex process which necessarily demands sophisticated and sensitive analytical techniques for the proper identifications of a wide range of chemical compounds. It was found very advantageous and useful to combine gas chromatography (GC) with mass spectrometry (MS) in the context of organic residue analysis in previous years. GC-MS is the preferred technique for the analysis of lipid extracts of visible and absorbed residues obtained by solvent extraction (Evershed, 2008). The gas chromatograph separates a mixture, and the individual components pass directly into the mass spectrometer so that individual mass spectra can be recorded. The separation of the mixture's components is carried out by the GC and, at the same time, identification of the components is achieved by MS. Because it allows separation of many volatile and semi-volatile compounds, GC is very efficient although not always capable of selective detection. On the other hand, MS is found to be very efficient in selective detection although not always capable of proper separation (Sneddon et al., 2007).

The coupling of MS with GC makes it a powerful tool for investigation of the complex and aged mixtures of organic molecules encountered as constituents of historical and archaeological objects. MS can detect the molecular formula of the components of the sample, usually ionized by the loss of an electron during collision with the high energy electrons inside the electron beam. The subsequent decomposition of the molecular ion forms the ion fragments providing significant information about the molecular structure (Sneddon et al., 2007).

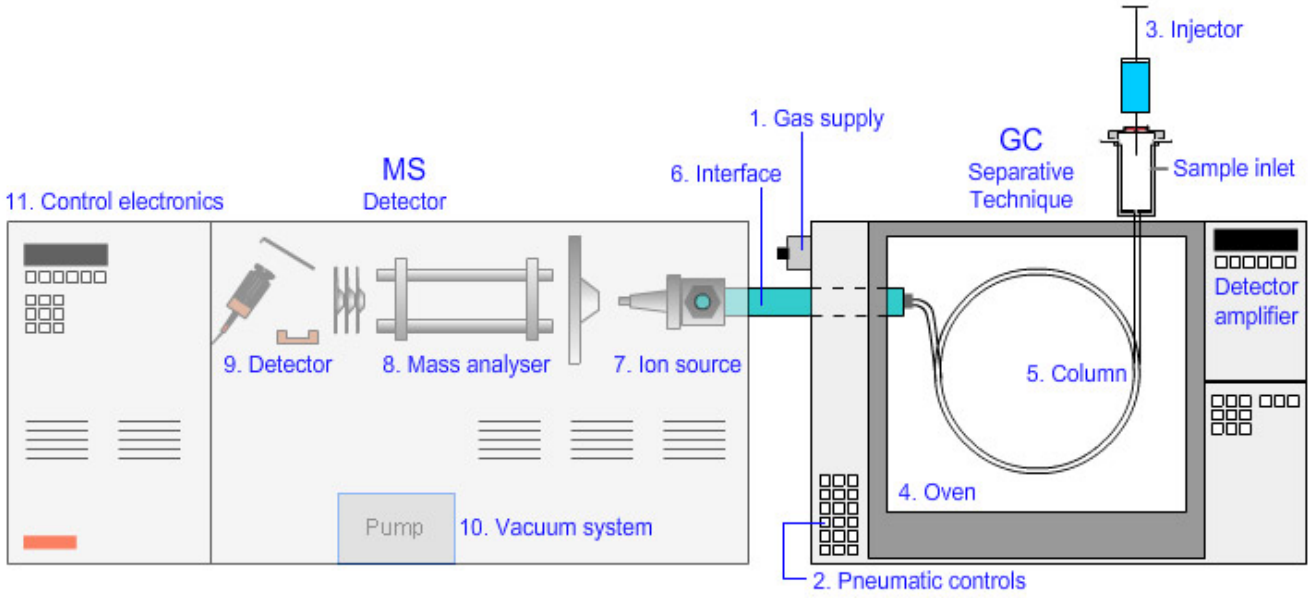


Figure 14 Schematic set up of gas chromatography mass spectrometry (GC-MS) (Source: https://www.chromacademy.com/resolver-november2010_understanding_gcms_part_1.html)

The sample can be in solution for injection into the GC. This can simply be done by dissolution in a solvent, typically dichloromethane, or extraction using organic solvents (Sneddon et al., 2007). In GC-MS analytical technique, the materials of investigation are introduced at a temperature around 300ºC in the GC system. Subsequently the effluent elutes from the GC and enters the MS, typically equipped with an electron ionization source, for the generation of ions. At this stage, the phase is bombarded with a stream of electrons, causing them to fragment. The mass of the fragment divided by the charge is presented as mass-to-charge ratio (m/z) (Sneddon et al., 2007).

Finally, the ions reach the mass analyser, which can be a quadrupole, an ion trap or a time-of-flight mass analyser. The configuration of the GC-MS equipment used for this work includes a quadrupole as mass analyser. For focusing each fragment through a slit into the detector, a group of four electromagnets (quadrupole) is used, and only certain fragments are selected to reach the detector. The quadrupoles are essentially programmed by a computer and cycle these fragments one at a time (scan) until the range of m/z is recovered. This phenomenon produces a mass spectrum as a result, which is a graph of signal intensity (abundance) versus m/z ratios (essentially molecular weight). Every and each compound has a unique fingerprint and the software used for data treatment includes a library of spectra for the unknown compounds.

In summary, GC-MS is an ideal technique for qualitative and quantitative determination of volatile and semi-volatile organic compounds in a wide variety of samples, with a detection limit as low as sub-nanogram (Sneddon et al., 2007).

2.2 LA-ICP-MS analytical technique and its applicability for lead content analysis

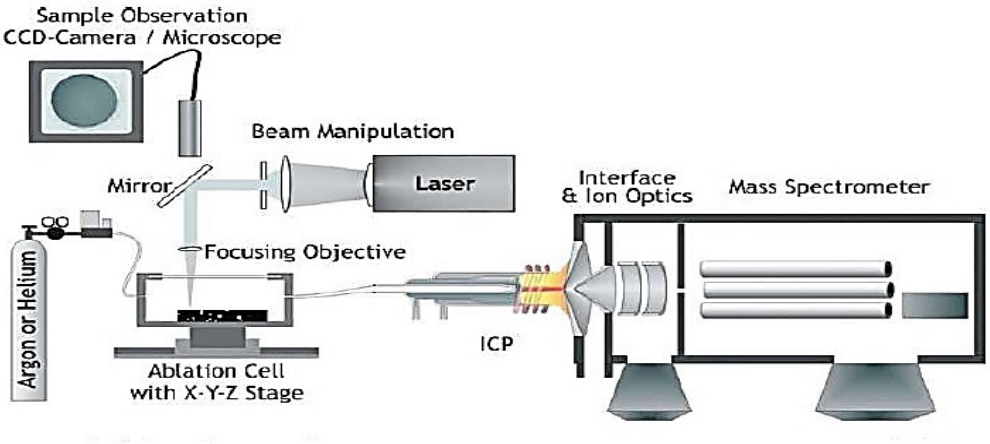
When a laser ablation (LA) system is used to vaporize the materials from a solid sample and analyze them by an inductively coupled plasma (ICP) based MS system, the total procedure can be termed as LA-ICP-MS analysis. The basic objective is to combine a direct solid introduction system capable of high spatial resolution with the highest sensitivity detector available for inorganic analysis. This hyphenated technique has developed into an exceptional instrument, which helped for the direct investigation of the elemental composition in solid samples (Giussani et al., 2009).This method was found very advantageous in the early 1990's for the characterization of archaeological materials as it involves minimum destructive procedures. At the beginning of its development phase, it was found very useful for the chemical characterization of pottery, chert, obsidian and painted-glazed surfaces (Speakman et al., 2002).

In the earlier stage of ICP-MS, samples were introduced into the plasma in liquid form, which required solid samples to get digested by heat and strong acid interaction. This digestion process was found very time consuming, complicated, unpleasant and involved many dangers as well. In that scenario, archaeological pottery and many lithic materials required hydrofluoric acid digestion. LA was found as an alternative to the digestion of the samples for ICP-MS analysis. In addition to the elimination of acidic digestion steps, LA also enabled ICP-MS as a very sensitive microprobe for the determination of a wide range of elements in the periodic table with a concentration < 1 ppm (Neff, 2012).

For conducting LA-ICP-MS the samples are placed inside the sample holder or laser cell where the ablation takes place. The cell is usually moveable in x, y, z axis and the sample observed by a microscope through the transparent window for setting the ablation position on desired spot. Ablation patterns such as lines, raster and spots are drawn on the samples. Afterwards the spot size, dwell time, scan speed and laser power which are known as characteristics of laser must be determined. Concerning the wavelengths issues, the fourth and fifth harmonic of the Nd‑YAG are usually applied to the investigation of cultural heritage objects. The area of ablation varies in size based on the sample matrix, but it is to mention that

usually smaller than 1000×1000 µm and less than 30 µm deep. Later the sample is introduced into the ICP-MS torch, where an argon gas plasma capable of sustaining electron temperature between 8000-10,000K is used for the ionization of the injected sample. Then the newly produced ions are passed through two stage interface system which is mainly designed for the transition of ions from atmospheric pressure to the vacuum chamber of ICP-MS. For the proper acceleration of the ions entered mass spectrometer are introduced with high voltage firstly and then pass through a series of focusing lenses, an electrostatic analyzer and at the end with a magnet. Depending on the variation to the strength of the magnet ions get separated according to mass/charge ratio and then leap over through a silt presented to the detector which is only capable of recording a very small atomic mass range at a certain given time. It is possible to scan the entire mass range within a very short period time by altering or varying the instrumental settings (Speakman et al., 2002).

It is possible to target the laser on spots which are very tiny in diameters (possibly 5 µm). The combination of small spot size and the high sensitivity of magnetic-sector ICP-MS for a wide range of major, minor, and trace elements make LA-ICP-MS a very powerful microprobe analysis. In addition, it is obvious that laser ablation is virtually non-destructive to most samples considering that the ablated areas are often indistinguishable with a naked eye.



D. Günther, B. Hattendorf, TrAC (2005), 24(3), 255-265.

Figure 15 Schematic set up of LA inductively coupled plasma mass spectrometry (LA ICP MS) (Source: https://www.futureocean.org/de/forschung/forschungsplattformen/forschungsausstattung/instrumentation/detail.php?id=2807)

In this research for the determination of lead (Pb) content in the depth profile of ceramic (Lusitana 9/ Sado 2 type amphorae sherds) LA-ICP-MS analysis will be applied. This technique is considered as a microprobe analytical technique for its high sensitivity and detection level. The imaging protocol by LA-ICP-MS for elemental distribution in archaeological ceramics also seems to be valid. Focus will be given to correlate samples containing resinous substances, phenolic acid compounds and lead (Pb) content, which can yield information about the possibility of amphorae use for storing or transporting wine during its lifetime.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sampling

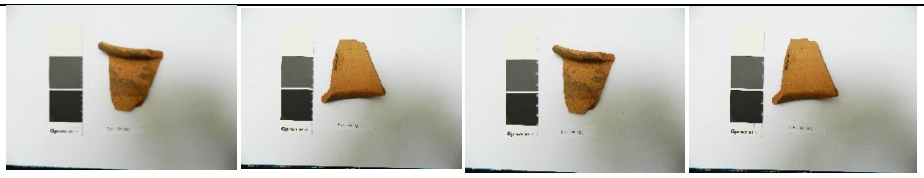
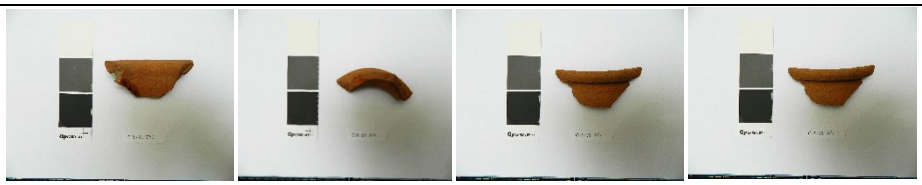
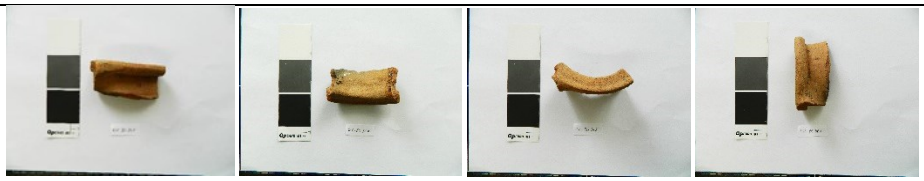
A set of twenty amphora sherds (Lusitana 9 / Sado 2 type) was selected for the GC-MS analysis of organic residues and wine biomarkers. For the LA-ICP-MS analysis, due to sample sizing and format, only twelve of these samples were cold mounted in epoxy resin (Epofix, Struers) and polished according to the classical methods. The set of twenty amphorae sherds was photographed, for record purposes. All samples mounted in epoxy resin were also photographed using a stereomicroscope.

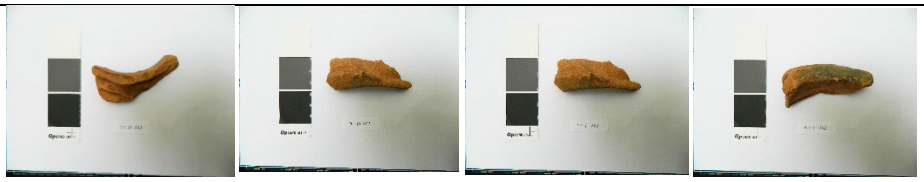
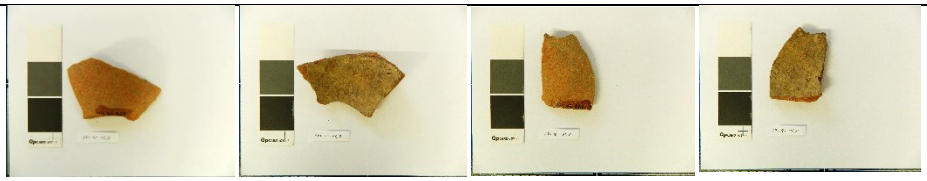
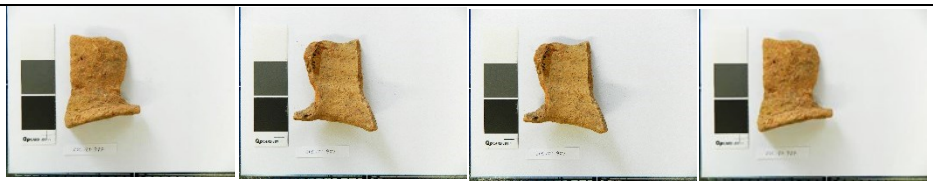
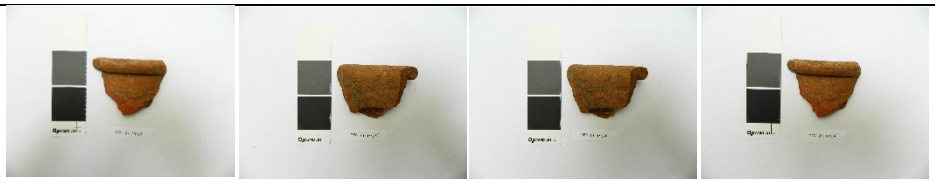
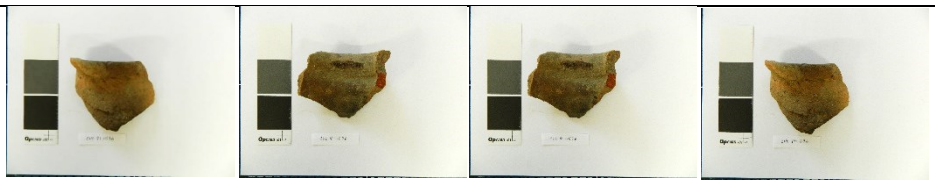
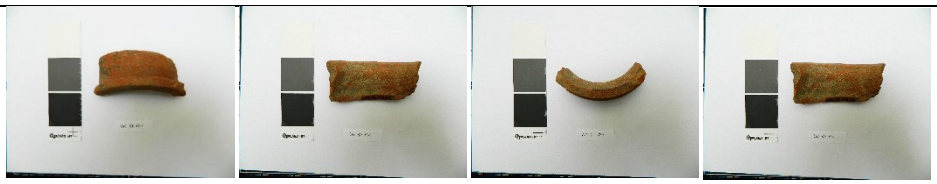
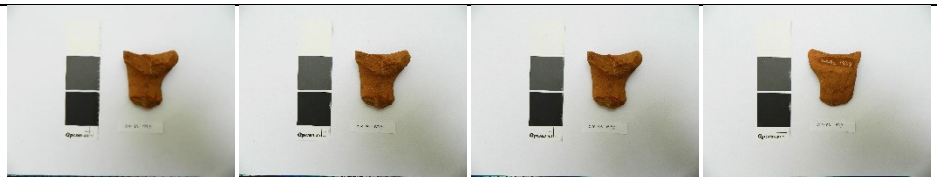
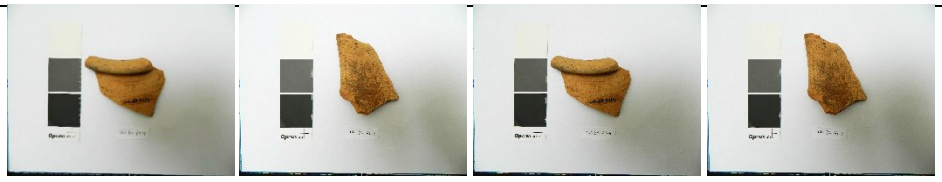
Table 2 Samples of Lusitana 9/ Sado 2 amphorae sherds collected from the archaeological site of São Cucufate and that were used for this study

Sample description			**Methodology**	
Site	**Year**	**Unit**	**Organic residue extraction and analysis**	**Lead isotopic analysis by LA-ICP-MS**
CUC	79	0645	√	
CUC	79	0986	√	√
CUC	80	0711	√	√
CUC	80	0767	√	√
CUC	81	0263	√	
CUC	81	0967	√	√
CUC	81	0977	√	√
CUC	81	1465	√	
CUC	81	1536	√	√
CUC	81	1841	√	

Sample description			Methodology	
Site	**Year**	**Unit**	**Organic residue extraction and analysis**	**Lead isotopic analysis by LA-ICP-MS**
CUC	82	1939	√	
CUC	82	5214	√	√
CUC	83	2696	√	√
CUC	83	3771	√	√
CUC	83	3793	√	√
CUC	83	4630	√	
CUC	83	5432	√	
CUC	84	4040	√	
CUC	84	5245	√	√
CUC	84	5483	√	√
Total			**20**	**12**

Table 3 Photographic record of the Lusitana 9 / Sado 2 amphorae sherds collected from the archaeological site of São Cucufate and that were used for this study

Sample ID	Images
Cuc. 79_645	
Cuc.79_986	
Cuc. 80_711	
Cuc. 80_767	

Sample ID	Images
Cuc. 81_263	
Cuc. 81_967	
Cuc. 81_977	
Cuc. 81_1465	
Cuc. 81_1536	
Cuc. 81_1841	
Cuc. 82_1939	
Cuc. 82_5214	

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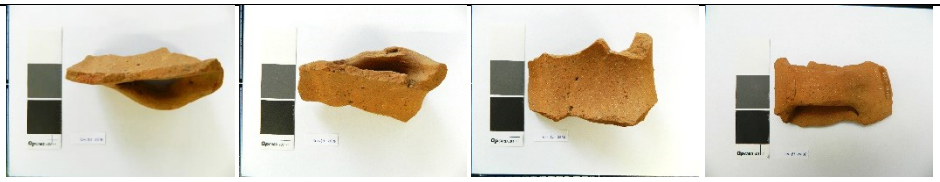
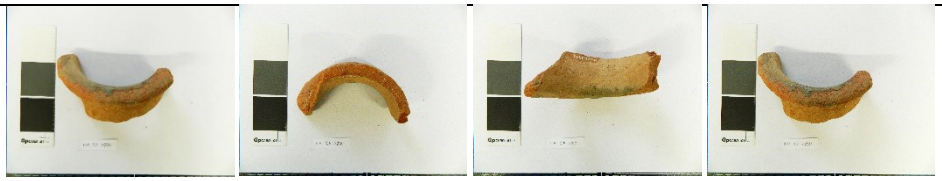
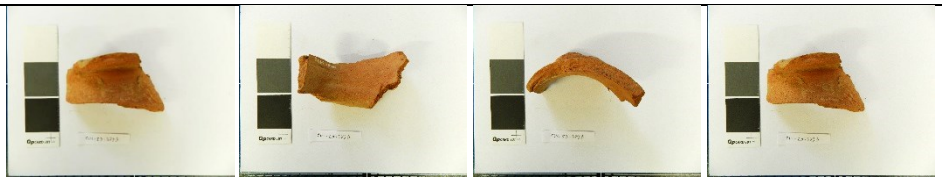
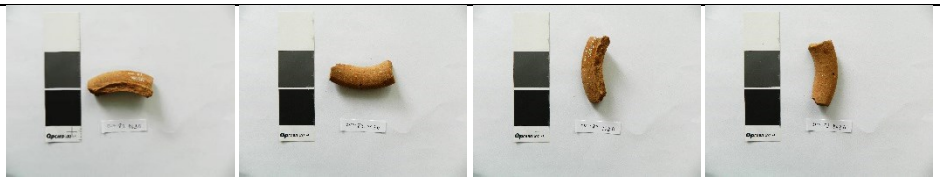
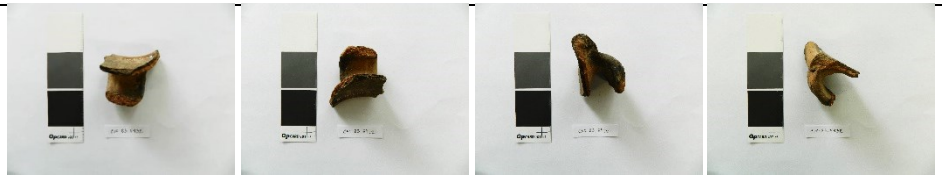
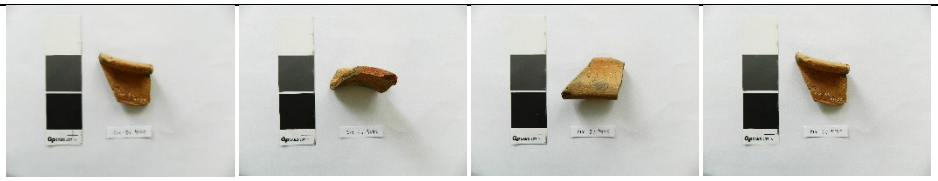
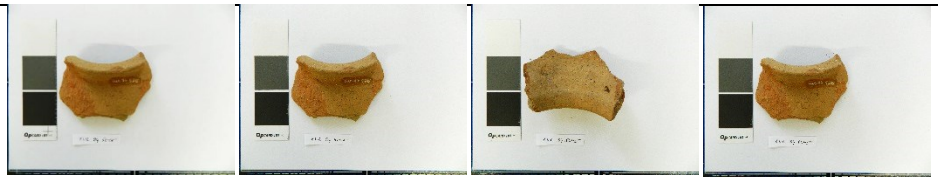
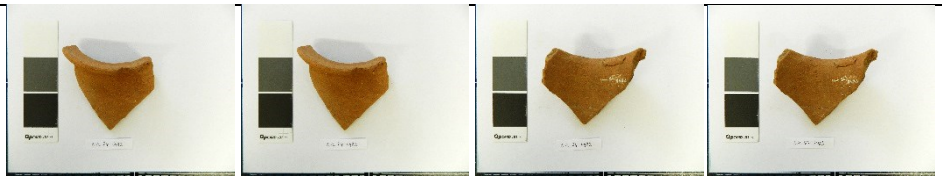
Cuc. 83_2696	
Cuc. 83_3771	
Cuc. 83_3793	
Cuc. 83_4630	
Cuc. 83_5432	
Cuc. 84_4040	
Cuc. 84_5245	
Cuc. 84_5483	

Table 4 Stereo microscopic images of the resin embedded samples selected for LA-ICP-MS analysis (magnification: 0.78x)

Sample ID	Image
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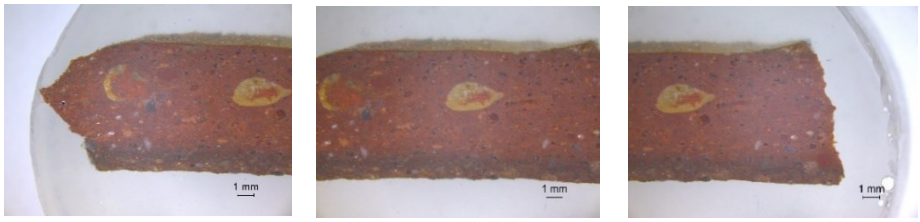
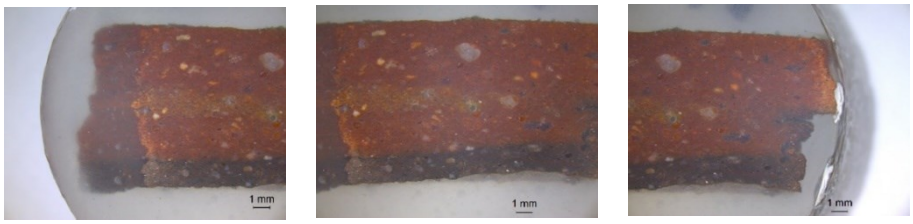
ceg-80.711	
ceg-80.767	
ceg-81.967	
ceg-81.977	
ceg-81.1536	

Sample ID	Image
ceg-82.5214	
ceg-83.2696	
ceg-83.3771	
ceg-83.3793	
ceg-84.5245	
ceg-84.5483	

3.2 Extraction of organic compounds from amphorae sherds

The drilling tool was used for cleaning the fragments of amphorae sherds which were selected for analysis. There were several marks of soil remains visible with unaided eyes, this drilling process helped removing those soil remains, and different types of contamination, during the burial process or handling of potsherds. Around 4-5 grams of sherds were detached using the cutter and grinded using a ball grinding mill for making powder of the samples. Mill container and balls were cleaned using chloroform/methanol solution (2:1, v/v) between each sample.

3.2.1 Total lipid extraction

The method of extraction was followed from previously published work of Mukherjee et al., 2008. Ten mL of $CHCl_3$: MeOH (2:1, v/v) were added to a 2g sample of powdered ceramics, together with 20µL of internal standard n-tetratriacontane ($1mg.mL^{-1}$). This was followed by a 15 min extraction in an ultrasonic bath and subsequent centrifugation (2500rpm, 15 min). Supernatant was removed, and procedure was repeated twice. The combined supernatants were dried at 40ºC under a light stream of N_2 to dryness and resuspended in 250µL of $CHCl_3$: MeOH (2:1, v/v). 50µL of this solution (total lipid extract, TLE) were collected in 1.5 mL glass vial and dried at 40ºC under a light stream of N_2. The total lipid extract (TLE) was re-dissolved in n-hexane and derivatized with N,O-bis(trimethylsilyl) trifluoroacetamide containing 1% of trimethylchlorosilane (BSTFA+ 1% TMCS) in a microwave oven (700W, 30 seconds). BSTFA excess was removed under light stream of N_2 and the derivatized TLE was dissolved in n-hexane and analyzed by GC/MS.

3.2.2 Wine biomarker extraction

The method of extraction was followed from previously published work of Pecci et al., 2016. 500 mg of the sample were extracted with KOH (1 M, 3 mL) in water, in a sonicated bath at 70ºC for 90 minutes. After cooling and centrifugation (2500rpm, 10 minutes), the supernatant was recovered and acidified with approximately 15 drops of HCl. Then, 3 mL of ethyl acetate was added to the acidified solution and mixed by vortexing for 2 minutes. After vortexing, the phases were separated by centrifugation

(2500rpm, 10 minutes), and the supernatant was removed. Ethyl acetate extraction was repeated two more times. The combined supernatants were dried completely using a gentle stream of nitrogen. Later, 25 µL of BSTFA was added to the final dried extract from the previous step. Derivatization was done in microwave (700W, 30 seconds), and BSTFA excess was removed under light stream of N_2. 100 µL of hexane and 5 µL of n-tetratriacontane (internal standard) were added, and 1 µL of sample was injected in the GC/MS system.

3.3 Gas chromatography-mass spectrometry (GC-MS)

A Shimadzu GC2010 gas chromatographer coupled to a GCMS-QP2010 Plus Mass Spectrometer was used. The gas chromatographer was equipped with a Zebron column ZB-5HT 0.25 mm × 15 m (0.10 µm film thickness, df). The sample (1 µL) was injected in split less mode for a sampling time of 1 minute with the injector temperature set at 250ºC and a helium column flow of 1.5 $mL.min^{-1}$. The GC oven temperature program was set at 50ºC for 2 min, then ramped to 300ºC at 10ºC/min, followed by a 5 min isothermal period. The temperature was increased again to 400ºC at 10ºC/min and held during 5 min. The mass spectrometer was operated in EI mode (70 eV). Ion source temperature was set at 240°C and interface temperature was maintained at 280°C. The mass spectrometer was scanned from 40 to 850 m/z. Peak assignment was done using the National Institute of Standards and Technology (NIST) mass spectra library, through AMDIS software.

3.4 Laser Ablation - Inductively Coupled Plasma - Mass Spectrometry (LA-ICP–MS)

The LA-ICP-MS method was chosen as the most suitable one given the fact that the aim was to check the lead content in depth profile of samples: the inside part of the amphorae should be more enriched in lead than the outside. Analysis was performed using an Agilent 8800 ICP-MS Trip Quad coupled to a CETAC LSX - 213G^{2+}laser ablation system. The equipment was calibrated prior to the analysis with NIST 612. Elemental fractionation was monitored using the $^{238}U/^{232}Th$ (≅102%) and the oxide formation was evaluated using $^{248}ThO/^{232}Th$ (< 0.15% ratio). For the present study, ICP-MS was performed in TRA mode (Time Resolved Analysis) operating with the scan type single quad mode. The elements monitored and their integration times, as well as the ICP conditions are showed in the following table.

Table 5 LA-ICP-MS analysis conditions

Acquisition Mode	TRA (Time Resolved Analysis)
Scan Type and Tune Mode	Single quad/ No gas
Plasma Parameters	
RF Power	1550 W
RF Matching	1.4 V
Sample Depth	6.5 mm
Dilution Gas (Ar)	0.60 L/min
Plasma Gas (Ar)	15 L/min
Drawn method parameters	
Spot size	50µ
Space between line	30
Energy	100%
Shot Frequency	20hz
Scan rate	100µ/sec
No. of line	15-30
Dwell time	20ms

The percentage of recovery of all elements was performed to check the accuracy of the measurements, which showed to be between 95 and 103%. iQuant® software was used to convert the intensity of the elements into an elemental distribution map.

3.5 Summary of research materials and methods

Materials and methods are considered as a very vital part of any kinds of research associated with scientific analytical techniques. The quality of all research and its outcomes depends mostly on the adopted methodological approaches and prudence during the time of application. Considering the importance of this part of research special care was taken from the beginning of this section. A set of twenty amphora sherds (Lusitana 9 / Sado 2 type) were selected for the analysis of organic residues by total lipid extraction. Lipids and other non-polar compounds, such as sterols, long chain alcohols and alkanes, are usually recovered from archaeological ceramics using a mixture of organic solvents like chloroform: methanol (2:1, v/v), which was proved very useful in several previous studies on similar field and applied in this study. More polar chemical compounds tend to interact with the ceramic compounds and degrade, which demands much more harsh extraction. Regarding the more polar nature of phenolic acids (tartaric acid, syringic acid, hydrocinnamic acid, vanillic acid) present in wine, alkaline extraction (KOH) method was applied. Before applying this protocol for wine biomarker extraction

several methods and their applicability were studied. GC and GC-MS are the preferred techniques for the analysis of lipid extracts of visible and absorbed residues obtained by solvent extraction. LA-ICP-MS was chosen for checking the lead (^{238}Pb) contents distribution in the depth profile.

CHAPTER 4: RESULTS - FINDINGS AND DISCUSSION

4.1 Analysis of organic residues in amphorae by GC/MS

Unglazed ceramic containing porous structure is very favorable for the entrapment of liquid or semi liquid materials being stored in the ceramic's lifetime and it partially protects the organic molecules from degradation and water leaching during the whole time of burial process. Those entrapped materials inside the porous structure leave behind a chemical fingerprint which can be recovered nowadays using sophisticated methodological approaches. Lipids and other non-polar compounds, such as sterols, long chain alcohols and alkanes, are usually recovered from archaeological ceramics using a mixture of organic solvents like chloroform: methanol (2:1, v/v) (Mukherjee et al., 2008). Slightly more polar compounds, such as unsaturated fatty acids and products of their degradation (diacids and hydroxyacids), are usually poorly recovered with such methodology. More polar organic compounds are usually likely to interact strongly with the structural components of ceramic. They do this interaction by electrostatic and dipole-dipole chemical interactions, which are a very common scenario in that perspective. It requires much more harsher conditions for recovering more polar compounds. Researchers have proved that strong basic conditions (KOH and methanol) (Regert et al., 1998) can favor the recovery of highly bound compounds and, more recently, a procedure using acidic conditions (H_2SO_4 and methanol) (Correa-Ascencio & Evershed, 2014) was also proposed.

It is already being proved that the harsher extraction condition can lead the way to the hydrolysis of any ester molecules present in the samples. For instance, TAGs of plant and animal fats or the wax esters of the beeswax are more likely vulnerable to degradation, with the concomitant information loss (Historic England, 2017). Considering the above-mentioned situation, the samples were extracted using organic solvent extraction method which is discussed in Chapter 3: Materials and Methods section.

4.1.1 Analysis of total lipid extract

Lipids are biomolecules soluble in nonpolar solvents. Non-polar solvents are typically hydrocarbons used to dissolve other naturally occurring hydrocarbons and lipid molecules that do not or are not easily dissolve in water, including fatty acids, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides, and phospholipids. Scientists sometimes broadly define lipids as hydrophobic or amphiphilic small molecules; the amphiphilic nature of some lipids allows them to form structures such as vesicles, multilamellar/unilamellar liposomes, or membranes in an aqueous environment. Most of the plant and animals' cells mainly a combination of lipids, carbohydrates and proteins (Fahy et al., 2009).

Triacylglycerols (TAGs) are a form of lipids, chemically structured with a glycerol molecule bound to three fatty acids. Previous studies show that TAGs are making more than 95% of lipids in our diet, which is found in plant and animal products such as plant or vegetable oils, nuts, seeds, dairy, fishes and meats. For all kinds of organic residue analysis, the constituents (fatty acids) of TAGs are considered as the most abundant compounds, although many fatty acids are not biomarkers (Historic England, 2017).

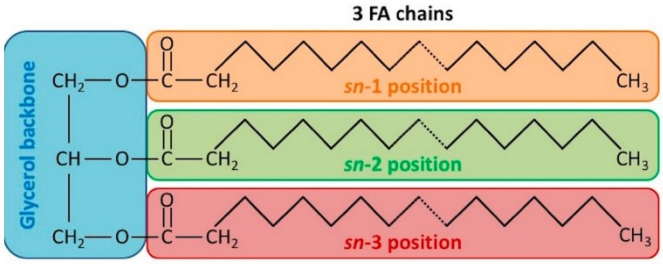


Figure 16 Triacylglycerol

Natural waxes produced by the insects, like beeswax, and the epicuticular waxes (present in plant oils) are products that can be revealed in organic residue analysis of ancient vessels (Historic England, 2017). Resins, tars and bitumen are natural products mainly used for non-dietary purposes that can also be identified by conducting residue (visible or absorbed) analysis. In previous studies it was found that these non-dietary natural products are mainly used as sealants, adhesives or waterproofing during the manufacture of the pottery (Historic England, 2017).

Before going deeper into the analysis of several chemical compounds of this research it is to mention that from the time of excavation the storage of the collected amphorae sherds from the archaeological

site of São Cucufate, samples went through an extensive process of documentation. This necessarily results in the contaminations of the samples by touching, cleaning, washing, wrapping with plastic bags and so on. In several samples, we have found the chemical compounds associated with the plastic contamination, which are likely diethyl phthalate, isobutyl phthalate, dibutyl phthalate, benzyl butyl phthalate, ethylhexyl phthalate and methyl 2-ehylehexyl phthalate (Oudemans & Boon, 1993).

There has been a significant number of chemical compounds found in the chromatograms presented in two tables below, which likely are due to the use and reuses of amphorae for different purposes during its lifetime. Tables 6 and 7 contain the complete list of compounds identified in the total lipid extracts for all samples. For an easier interpretation of results, Table 8 present a summary of all results.

Table 6 Peak assignment of the compounds identified in samples 79_645, 79_986, 80_711, 80_767, 81_1465, 81_1536, 81_1841, 81_263, 81_967 and 81_977

RT	Name	Type of compound	79_645	79_986	80_711	80_767	81_1465	81_1536	81_1841	81_263	81_967	81_977
4.22	Undecane	n-alkane					√		√			
6.45	Dodecane	n-alkane	√		√			√	√			√
6.89	Glycerol, tris(trimethylsilyl) ether	Glycerol				√						
6.91	Tridecane	n-alkane	√	√	√		√		√		√	
7.81	Nonanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√		√	√	√		√	√	
8.19	Tetradecane	n-alkane	√		√		√		√	√	√	√
9.39	Pentadecane	n-alkane	√		√		√	√	√	√	√	
10.27	1-Dodecanol, trimethylsilyl ether	Fatty alcohol	√	√					√	√		
10.50	Diethyl pthalate	Phthalate						√	√			
10.53	Hexadecane	n-alkane	√	√	√		√	√	√	√	√	√
11.18	Dodecanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√		√	√		√	√	√
11.61	Heptadecane	n-alkane	√	√		√	√	√	√	√		√
12.64	Octadecane	n-alkane	√	√		√	√		√	√	√	
12.94	Isobutyl trimethylsilyl phthalate	Phthalate		√								
13.23	Tetradecanoic acid, trimethylsilyl ester	Saturated fatty acid		√								√
13.26	Isobutyl phthalate	Phthalate	√	√	√	√	√	√	√	√	√	√
13.67	Nonadecane	n-alkane		√	√	√			√	√	√	
13.88	Hexadecanoic acid, methyl ester	Saturated fatty acid	√	√	√	√	√	√	√	√	√	√
14.16	Dibutyl phthalate	Phthalate	√	√	√		√	√	√		√	
14.19	Pentadecanoic acid, trimethylsilyl ester	Saturated fatty acid		√								
14.28	1-Hexadecanol, trimethylsilyl ether	Fatty alcohol	√	√	√	√						
14.62	Eicosane	n-alkane			√							
15.05	Hexadecanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√		√	√	√	√	√	
15.71	Octadecanoic acid, methyl ester	Saturated fatty acid	√	√		√		√	√	√	√	
16.04	1-Octadecanol, trimethylsilyl ether	Fatty alcohol	√	√	√	√	√	√	√	√		√

RT	Name	Type of compound	79_645	79_986	80_711	80_767	81_1465	81_1536	81_1841	81_263	81_967	81_977
16.38	Docosane	n-alkane		√						√		
16.55	Oleic acid, trimethylsilyl ester	Unsaturated fatty acid		√								√
16.76	Octadecanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√	√				√	√	√
17.06	Pimaric acid, trimethylsilyl ester	Diterpenoid					√					
17.32	Isopimaric acid, trimethylsilyl ester	Diterpenoid			√		√					
17.47	Benzyl butyl phthalate	Phthalate		√			√					
17.71	1-Eicosanol, trimethylsilyl ether	Fatty alcohol		√		√	√			√		
17.74	Dehydroabietic acid, trimethylsilyl ester	Diterpenoid				√	√					
17.99	Abietic acid, trimethylsilyl ester	Diterpenoid			√		√					
18.01	Tetracosane	n-alkane			√				√	√		
18.13	1-Monomyristin trimethylsilyl ether	Monoacylglycerol (MAG)				√					√	
19.03	Ethylhexyl phthalate	Phthalate	√	√	√	√	√	√	√	√	√	√
19.16	Dehydroabietic acid, methyl ester	Diterpenoid		√	√							
19.40	2-Monopalmitin trimethylsilyl ether	Monoacylglycerol (MAG)			√	√		√		√	√	√
19.48	Hexacosane	n-alkane							√	√		
19.58	1-Monopalmitin trimethylsilyl ether	Monoacylglycerol (MAG)	√		√	√	√	√	√	√	√	√
20.20	Heptacosane	n-alkane							√			
20.94	1-Monostearin trimethylsilyl ether	Monoacylglycerol (MAG)	√	√	√	√	√	√	√	√	√	√
21.60	Nonacosane	n-alkane		√								
23.01	Cholesterol trimethylsilyl ether	Animal origin sterol		√								
24.61	Tetratriacontane	n-alkane, Internal standard	√	√	√	√	√	√	√	√	√	√

Table 7 Peak assignment of the compounds identified in samples 82_1939, 82_5214, 83_2696, 83_3771, 83_3793, 83_4630, 83_5432, 84_4040, 84_5245 and 84_5483

RT	Name	Type of compound	82_1939	82_5214	83_2696	83_3771	83_3793	83_4630	83_5432	84_4040	84_5245	84_5483
4.22	Undecane	n-alkane				√						
6.45	Dodecane	n-alkane	√				√					

RT	Name	Type of compound	82_1939	82_5214	83_2696	83_3771	83_3793	83_4630	83_5432	84_4040	84_5245	84_5483
6.89	Glycerol, tris(trimethylsilyl) ether	Glycerol										
6.91	Tridecane	n-alkane	√			√			√	√	√	√
7.81	Nonanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√	√		√			√	√
8.19	Tetradecane	n-alkane	√	√	√	√	√	√	√	√	√	√
9.39	Pentadecane	n-alkane				√	√	√	√	√	√	√
10.27	1-Dodecanol, trimethylsilyl ether	Fatty alcohol			√				√			√
10.50	Diethyl pthalate	Phthalate, plastic contamination			√				√		√	
10.53	Hexadecane	n-alkane	√	√		√	√	√	√	√	√	
11.18	Dodecanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√	√		√	√	√	√	√
11.61	Heptadecane	n-alkane		√	√	√	√	√			√	√
11.92	Decanoic acid, methyl ester	Saturated fatty acid								√		
12.39	1-Tetradecanol, trimethylsilyl ether	Fatty alcohol									√	
12.64	Octadecane	n-alkane	√		√	√	√		√		√	√
12.94	Isobutyl trimethylsilyl phthalate	Phthalate, plastic contamination										
13.23	Tetradecanoic acid, trimethylsilyl ester	Saturated fatty acid		√	√							√
13.26	Isobutyl phthalate	Phthalate, plastic contamination	√	√	√	√	√	√	√	√	√	√
13.66	Heptadecane	n-alkane								√		
13.67	Nonadecane	n-alkane			√	√						√
13.88	Hexadecanoic acid, methyl ester	Saturated fatty acid		√	√	√	√		√	√	√	√
14.16	Dibutyl phthalate	Phthalate, plastic contamination	√	√	√		√			√	√	√
14.19	Pentadecanoic acid, trimethylsilyl ester	Saturated fatty acid									√	
14.28	1-Hexadecanol, trimethylsilyl ether	Fatty alcohol		√	√						√	√
14.62	Eicosane	n-alkane		√			√			√	√	√
15.05	Hexadecanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√	√	√	√	√	√	√	√
15.62	Methyl 2-ehylehexyl phthlate	Phthalate, plastic contamination			√	√		√				
15.71	Octadecanoic acid, methyl ester	Saturated fatty acid			√			√		√		√
16.04	1-Octadecanol, trimethylsilyl ether	Fatty alcohol	√	√	√	√	√	√	√	√	√	√

RT	Name	Type of compound	82_1939	82_5214	83_2696	83_3771	83_3793	83_4630	83_5432	84_4040	84_5245	84_5483
16.38	Docosane	n-alkane										
16.55	Oleic acid, trimethylsilyl ester	Unsaturated fatty acid	√	√	√	√		√			√	
16.76	Octadecanoic acid, trimethylsilyl ester	Saturated fatty acid	√	√	√	√	√	√	√		√	√
17.06	Pimaric acid, trimethylsilyl ester	Diterpenoid			√		√	√				
17.32	Isopimaric acid, trimethylsilyl ester	Diterpenoid			√	√	√	√				
17.47	Benzyl butyl phthalate	Phthalate, plastic contamination										
17.71	1-Eicosanol, trimethylsilyl ether	Fatty alcohol	√	√		√	√			√		
17.74	Dehydroabietic acid, trimethylsilyl ester	Diterpenoid	√		√	√	√		√			
17.99	Abietic acid, trimethylsilyl ester	Diterpenoid			√	√	√	√				
18.01	Tetracosane	n-alkane						√				
18.13	1-Monomyristin trimethylsilyl ether	Monoacylglycerol (MAG)			√							√
19.03	Ethylhexyl phthalate	Phthalate, plastic contamination	√	√	√	√	√	√	√	√	√	√
19.16	Dehydroabietic acid, methyl ester	Diterpenoid										√
19.40	2-Monopalmitin trimethylsilyl ether	Monoacylglycerol (MAG)	√		√						√	√
19.48	Hexacosane	n-alkane						√				√
19.58	1-Monopalmitin trimethylsilyl ether	Monoacylglycerol (MAG)	√	√	√	√	√	√	√	√	√	√
20.20	Heptacosane	n-alkane										
20.62	1-Tetracosanol, trimethylsilyl ether	Fatty alcohol		√			√		√			
20.72	2-Monostearin trimethylsilyl ether	Monoacylglycerol (MAG)										√
20.94	1-Monostearin trimethylsilyl ether	Monoacylglycerol (MAG)	√	√	√	√	√	√	√	√	√	√
21.60	Nonacosane	n-alkane										
21.94	1-Hexacosanol, trimethylsilyl ether	Fatty alcohol		√					√			
23.18	1-Octacosanol, trimethylsilyl ether	Fatty alcohol		√								
24.35	1-Triacontanol, trimethylsilyl ether	Fatty alcohol		√								
24.61	Tetratriacontane	n-alkane, Internal standard	√	√	√	√	√	√	√	√	√	√

The assignment of a specific source or constituent of a residue based on the presence of a particular biomarker component or mixture of components demands a high degree of rigor wherein consideration of the nature of other constituents of the residue may lead to the hypothesis of a putative source being rejected (Evershed, 2008).

Long chained alkanes such as tridecane (C13), tetradecane (C14), pentadecane (C15), hexadecane (C16), heptadecane (C17), octadecane (C18), nonadecane (C19), eicosane (C20), docosane (C22), tetracosane (C24), hexacosane (C26), heptacosane (C27) and nonacosane (C29) are very common chemical compounds for almost all the sample revealed in the chromatograms, which could be a matter of fact indicating the contact with the plant remains of amphorae. Author McGovern in his studies (on the year 2004) emphasized the presence of beeswax based on the presence of C23, C25, C27, C29, C31 and C33 n-alkanes which was nullified as the gas chromatographic profile showed a textbook n-alkane distribution characteristic of petroleum, wherein both odd and even carbon number homologues were present at similar abundance. Thus, consideration of the entire n-alkane complement, rather than selected components necessarily yields that these compounds do not unambiguously derive from beeswax. However, the report of Namdar et al. (in press) based an assignment of beeswax on similar n-alkane distributions, asserting that they can derive from heated-treated beeswax. Where such ambiguities exist firm conclusions as to the true nature of organic residues can only be reached once more robust supporting evidence has been obtained (the additional presence of hydroxy palmitate wax esters, 15-methoxypalmitic acid and C24, C26 and C28 saturated fatty acids) otherwise the question of origin of such distributions must remain open (Evershed, 2008). Long chain fatty acids can arise for example, from the presence of beeswax or *Brassicaceae* oils, but the lack of additional biomarkers precludes the identification of the source of the long chain fatty acids detected in the samples.

The table below is presented for the easier tracing of major compounds such as saturated and unsaturated fatty acids, n-alkanes, monoacylglycerols (MAG), diterpenoids, sterols and fatty alcohol present in the GC-MS chromatograms of samples.

Table 8 Distribution of several compound categories in the studied samples (summary of GC-MS results)

Sample	Compound category							
	n-alkanes	Saturated fatty acids	Unsaturated fatty acids	Fatty alcohols	Diterpenoids	MAG's	Plant sterols	Cholesterol
79_645	√	√		√		√		
79_986	√	√	√	√	√	√		√
80_711	√	√		√	√	√		
80_767	√	√		√		√		
81_1465	√	√		√	√	√		
81_1536	√	√		√		√		
81_1841	√	√		√		√		
81_263	√	√		√		√		
81_967	√	√				√		
81_977	√	√	√	√		√		
82_1939	√	√	√	√	√	√		
82_5214	√	√	√	√		√		
83_2696	√	√	√	√	√	√		
83_3771	√	√	√	√	√	√		
83_3793	√	√		√	√	√		
83_4630	√	√	√	√	√	√		
83_5432	√	√		√	√	√		
84_4040	√	√		√		√		
84_5245	√	√	√	√		√		
84_5483	√	√		√	√	√		

Sterols can be of much help in the investigation of pottery contents. Animal products contain high amounts of cholesterol, while plant materials produce large amounts of phytosterols and only minor amounts of cholesterol (Evershed, 2008). Among the 20 studied samples only one sample (79_986) yielded cholesterol (an animal origin sterol), which is unique and could mean a reuse of amphorae for animal origin fat products.

Major compounds, such as saturated fatty acids, unsaturated fatty acid, n-alkane, monoacylglycerol (MAG), diterpenoid, phthalate and fatty alcohol distribution in GC/MS chromatograms are presented in the figures 17, 18 and 19 below.

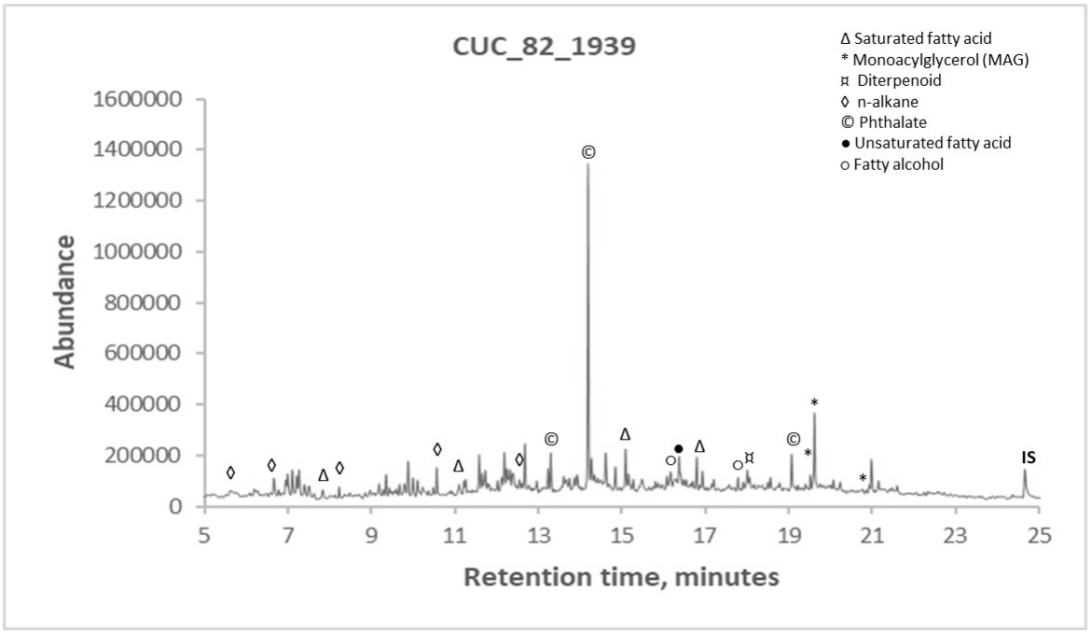


Figure 17 GC/MS chromatogram of sample 82_1939 (IS = internal standard, n-tetratriacontane)

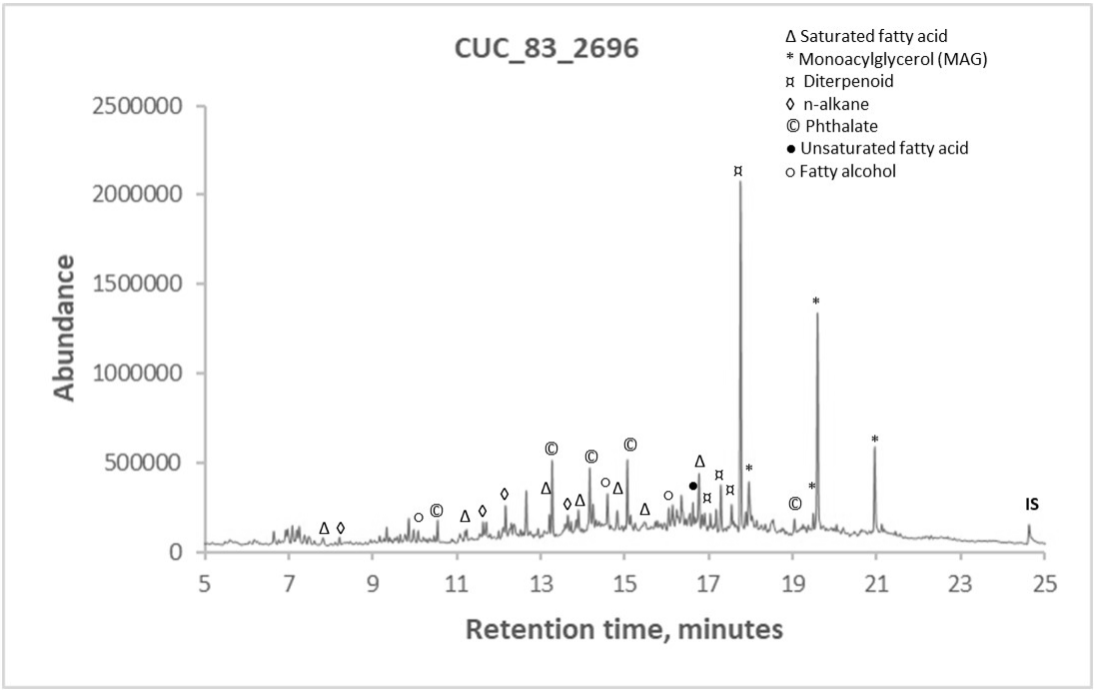


Figure 18 GC/MS chromatogram of sample 83_2696 (IS = internal standard, n-tetratriacontane)

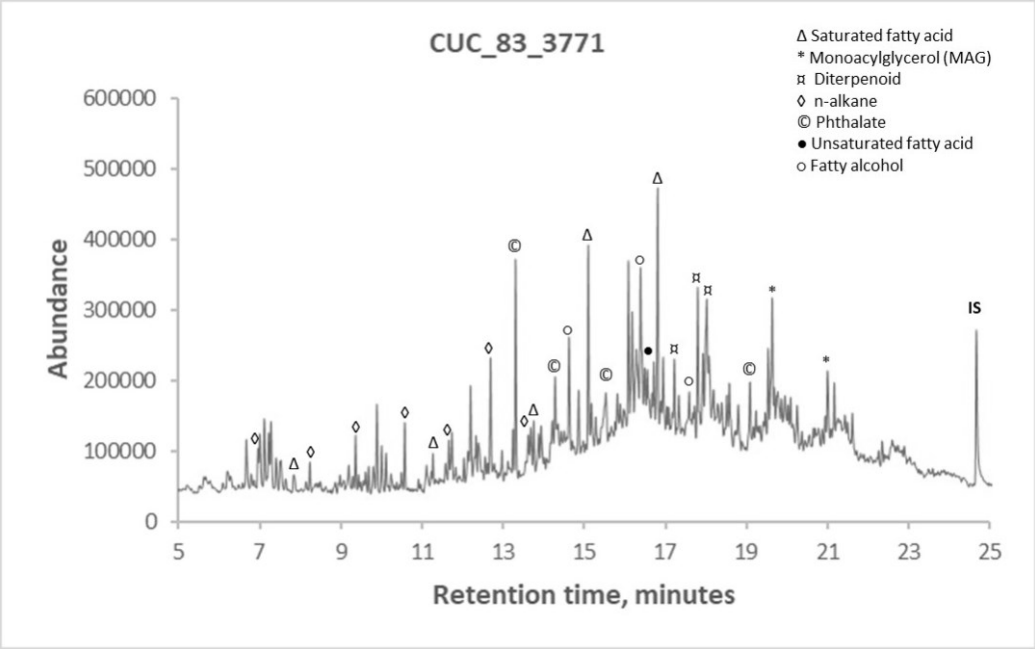


Figure 19 GC/MS chromatogram of sample 83_3771 (IS = internal standard, n-tetratriacontane)

Oleic acid (C18:1) has shown its presence in 8 of the 20 studied samples in this study. It is a frequently observed unsaturated fatty acid, albeit in widely varying abundance; this variability presumably reflects the source of food stuff and/or the level of oxidative/reductive damage experienced by the lipid (Evershed et al., 1991). Along with oleic acid, if palmitoleic acid (C16:1) and elaidic acid (C18:1 trans) were found, then it could be an indicator of certain plant oils. It is not possible to identify clearly olive oil presence in archaeological samples due to the lack of specific biomarkers for this oil. The findings of archaeological evidences of olive mills throughout the south of Portugal suggest the local production in the Lusitanian territory (Fabião, 2013). However, it is more likely that oil transported in different types of amphorae and found in Roman archaeological sites around the Lusitanian territory could be associated with the state distribution of olive oil for facilitating mining areas and some elites sustained in the rural and urban centers (Fabião, 1993). It's more logical that local produced olive oil was consumed locally and would not enter commercial routes.

Fresh olive oil has the following fatty acids present primarily within triglycerides: myristic acid (C14:0) <0.5%, palmitic acid (C16:0) 7.5-20%, palmitoleic acid (C16:1, cis-Δ) 0.3-3.5%, stearic acid (C18:0) 0.5-5%, oleic acid (C18:1, cis-Δ) 55-83%, linoleic (C18:2, cis-Δ) 3.5-21%, linolenic acid (C18:3, cisΔ) <1.0%, arachidic acid (C20:0) <0.6%, behenic acid (C22:0) <0.2%, lignoceric acid (C24:0) <0.2% (Aparicio et al., 2013). Fatty acid profile of olive oil in archaeological samples is very different, unless

the samples are extremely well preserved. Due to the environmental factors, fatty acids in olive oil are likely to be present in free form in the samples and highly prone to the oxidation, resulting in the formation of oxoacids, hydroxyacids and diacids (Copley et al., 2005; Hansel et al., 2011). Sterols can be a potential source for identification of olive oil; fresh oil provides a profile with high levels of β-sitosterol and Δ-avenasterol, and low levels of campesterol and stigmasterol, but these compounds are very unstable in nature and usually don't show their presence in archaeological samples (Evershed, 1993). The presence of additional unsaturated fatty acids (other than oleic acid) and their degradation products such as hydroxyacids and diacids was not detected, and the same happened for long chain esters (Colombini et al., 2009; Evershed, 2008).

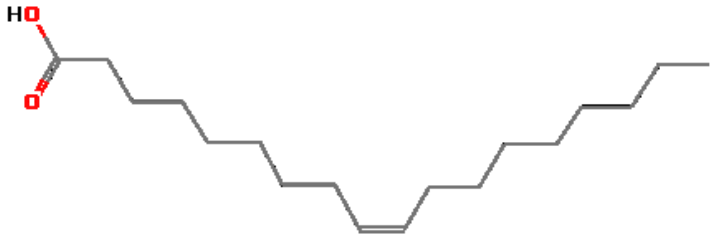


Figure 20 Oleic acid

Fatty alcohols like 1-dodecanol, 1-hexadecanol, 1-octadecanol, 1-eicosanol, 1-tetradecanol, 1-tetracosanol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, 1-triacontanol and their trimethylsilyl ether derivatives were identified in many samples which demands an explanation. The combination of straight-chain fatty acids and straight-chain primary alcohols is usually the result of the hydrolysis of the esters in epicuticular waxes (Beeston et al., 2006). In general, plant products can be associated with larger amounts of unsaturated fatty acids (or their degradation products, like α, ω-diacids, hydroxy acids or dihydroxy acids), additionally to the presence of a series of n-alkanes and n-alcohols, originating from plant epicuticular waxes (Evershed, 2008; Colombini & Modugno, 2009).

The fatty acid profile of animal and plant materials should be very different, but food processing and degradation during burial can complicate the identification of the original contents of the ceramic vessels (Evershed, 2008). Fatty acids have different solubility (short chain fatty acids are more water soluble) and have different susceptibilities to degradation (unsaturated fatty acids are more prone to degradation), both of which can substantially alter the initial fatty acid profile of an animal or vegetable product.

Despite this fact, some general rules apply and they can be followed in an attempt to infer on the ceramic's uses (Manhita et al., 2014).

Palmitic and stearic acid are the most commonly identified compounds in lipid residues. These usually indicate a degraded animal fat origin although they are also found in aquatic and plant fats. Individual fatty acid identification can also provide clues into the origin of the animal fat residues, as adipose fat and milk fat present different chromatographic profiles, with the milk fat being richer in short-chain fatty acids (Evershed et al., 2002). Among adipose fats, there are also differences between ruminant and non-ruminant fats, mainly due to differences in the food digestion process of these animals, which leads to the presence of the ruminant's fats of odd carbon numbered fatty acids (C15:0, C17:0 or C19:0) and several isomers of C18:1 (Δ 9, Δ11, Δ13, Δ14, Δ15, Δ16) (Evershed et al., 2002). When C15 and C17 are not with the isomers in C18:1, they are generally present due to microbial contamination of the samples (Evershed et al., 2002).

The trimethylsilyl ester of nonanoic acid (pelargonic acid, C9), decanoic acid (capric acid, C10) dodecanoic acid (lauric acid, C12), tetradecanoic acid (myristic acid, C14), hexadecenoic acid (palmitic acid, C16), pentadecanoic acid (C15) and octadecanoic acid (stearic acid, C18) were identified in a significant number of samples. Palmitic (C16:0) and stearic (C18:0) fatty acids are generally present in the extracts, and under normal conditions, chromatographic peak areas of C16:0 should be larger than C18:0, when in the presence of plant products (Manhita et al., 2015). However, C16:0 is more water soluble than C18:0, and it is frequent that C16/C18<1, even when one is almost sure of a plant origin for the archaeological residue, often requiring further analysis for confirmation (Colombini & Modugno, 2009; Steele et al., 2010).

Monoacylgycerols (MAGs) 1-monomyristin, 2-monopalmitin, 1-monostearin and 2-monostearin were yielded by almost every sample. Fats are the major components of organic residues in archaeological pottery and one of the most stable biomarkers. Both animal and plant fats are composed mainly by triglycerides in which three fatty acids are attached to a glycerol by ester bonds. Triacyl glycerides (TAGs) from plant and fish origin are richer in unsaturated fatty acids, while saturated fatty acids are predominant in TAGs from animal origin. During burial, the ester bonds in the triglycerides can be broken by hydrolysis with the release of the fatty acids. TAGs can degrade forming diacylglycerols (DAGs), when only one fatty acid group hydrolyses, or monoacylglycerols (MAGs), when two fatty acid

groups hydrolyse. Only the most well-preserved archaeological fats still contain TAGs, while MAGs are most often detected (Manhita et al., 2015).

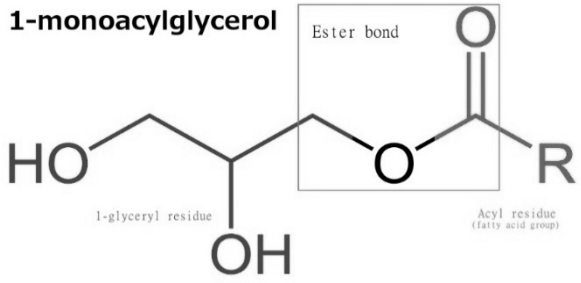


Figure 21 1-Monoacylglycerol

The diterpenoids pimaric, isopimaric, abietic and dehydroabietic acids were identified among the samples 81_1465, 82_1939, 80_711, 80_767, 79_986**,** 83_2696, 83_3771, 83_3793, 83_4630 and 84_5483. All these studied potsherds yield diterpenes that can be associated with the resins that exude from plants of the *Pinaceae* family (Colombini & Modugno, 2009). Diterpenoid acids, such as pimaric, isopimaric and abietic acids are known to be present in pine resin and clearly indicate their natural origin. However, dehydroabietic acid (DHA), 7-oxodehydroabietic acid and retene are the final products of dehydrogenation, demethylation or decarboxylation reactions that can either be related to the heating of those resins at high temperature or to alterations occurring during burial (Jerković et al., 2011).

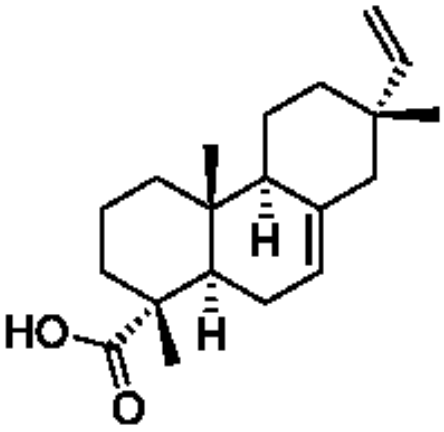


Figure 22 Isopimaric acid

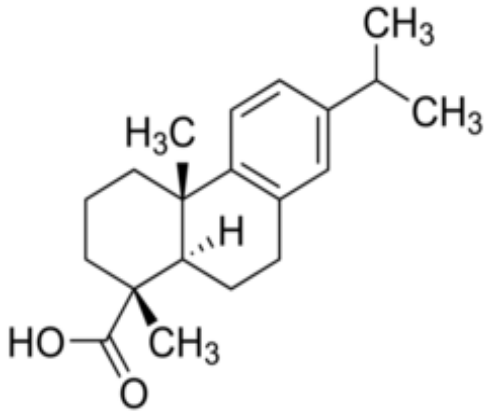


Figure 23 Dehydroabietic acid

According to Plinius and Columella, waterproofing of the ceramic material was done by the Romans to prevent product losses in the containers, using a material called pix (pitch) (Font et al., 2007). Pitch was obtained by subjecting resinous material or resinous wood to the elevated temperatures, resulting in a material mostly insoluble in water, but soluble in organic solvents. The use of resinous wood to produce pitch usually yields a unique biomarker, the methyl-dehydroabietic acid (MDHA), which results from the reaction at elevated temperatures of the methanol released from the wood with the resinous DHA (Jerković et al., 2011). The MDHA was absent from all the samples studied, while DHA was present in few of them.

Author Romanus *et al.* (2009) conducted an experimental procedure to prove the diffusion of viscous olive oil through the ceramic wall to the exterior layer without applying a layer of pitch and it was found that after 45 days, a significant amount of oil had diffused from the vessel base (Romanus et al., 2009). Romanus and co-workers reached to the conclusion that surface treatment with pitch layer was introduced in amphorae for avoiding oil waste during the transportation in late Roman period (Romanus et al., 2009). This phenomenon can be an explanation to the association of pitch and vegetable oils biomarkers.

Garnier *et al*. (2011) in their study also described the presence of pitch and oil biomarkers in Dressel 20 and African 1 oil amphorae (Garnier et al., 2011). Although Romanus *et al*. (2009) stated that olive oil could not dissolve the dried pitch, some authors defended the opposite (Garnier et al., 2011; Romanus et al., 2009; Peña, 2007 & Heron et al., 1988). They found that the oil would have mixed with pitch contents in course of time, leaving the oil with a particular smell and/or flavor. Pine resin flavor was likely appreciated in the Roman era, as it was added intentionally as flavoring agent of a highly prized resinous wine (McGovern, 2003).

Despite all the findings of wide range chemical compounds in the profile of samples it is quite hard to specify plant, animal or aquatic products as the original content. During the period of Roman time they used ceramic for several commodities, and studies of ceramic content can reveal the presence of oils, resins, fish and wines.

Plant oils can be collected from *Brassicaceae* family (mustard, turnip, sesame, linseed) and olives. The fatty acids composition of these fresh oils is found to be very complicated and different for each standard. Sesame and linseed oils are rich in polyunsaturated acids (C18:2 and C18:3) and consequently very prone to archaeological degradation or leaching, which can leave behind hydroxyacids and diacids as

biomarkers. These chemicals compounds were not found in the chromatograms. Like stated before, oleic acid (C18:1) was found in the chromatograms for 8 (79_986, 81_977, 82_1939, 82_5214, 83_2696, 83_3771, 83_4630 and 84_5245) of the 20 studied samples in this study which is a potential source to imply plant oil origin, although no other unsaturated fatty acid was detected. Even so, the presence of oleic acid (C18:1) could be an indicator of plant oils where other unsaturated fatty acids may degrade in course of time. No sample presents other fatty acids that can be used as biomarkers for some specific plant's oils, like erucic acid (C22:1 cis-Δ) for *Brassicaceae* oils, or ricinoleic acid (12-OH C18:1 cis-Δ) for castor oil (*Ricinus communis*) (Copley et al., 2005; Colombini et al., 2005).

Ceramic recycling in Roman period was a common phenomenon which complicated the identification of biomarkers in their contents. Amphorae could be reused for the same purposes after primary context of usage and it was not uncommon that it was used for carrying other commodities afterwards (Peña, 2007). According to the author Peña, there is some evidence that oil amphorae were in some occasions reused to carry wine, oil, fish products, cereals and vegetable (Peña, 2007).

4.1.2 Analysis of wine biomarkers

In previous years, different written sources, scholars and researchers mentioned about the possible use of Roman amphorae for carrying wine products, although it is very complicated to reveal the wine biomarkers (such as polyphenols, tartaric and syringic acids) for their highly hydrophobic and polar nature (Stern et al., 2008; Barnard et al., 2011). Although the diterpenoids pimaric, isopimaric, abietic and dehydroabietic acids were identified among 8 studied samples, it is probable that wine phenolic acids might not show their presence in all these 8 samples. This could complicate the validation of the hypothesis of using these amphorae as wine carrier vessels.

For the analysis of wine biomarkers, a set of 20 amphora sherds were chosen, the same set that was analysed with total lipid extraction. Before applying the harsh method of alkaline extraction (KOH) (Alessandra Pecci et al., 2013) to the archaeological samples, the same methodology was applied for an archaeological pottery sample that was in contact with red wine for 3 months in the laboratory. GC-MS analysis was used, and a set of phenolic acids were identified in the chromatograms, which are presented in the chromatogram and table below.

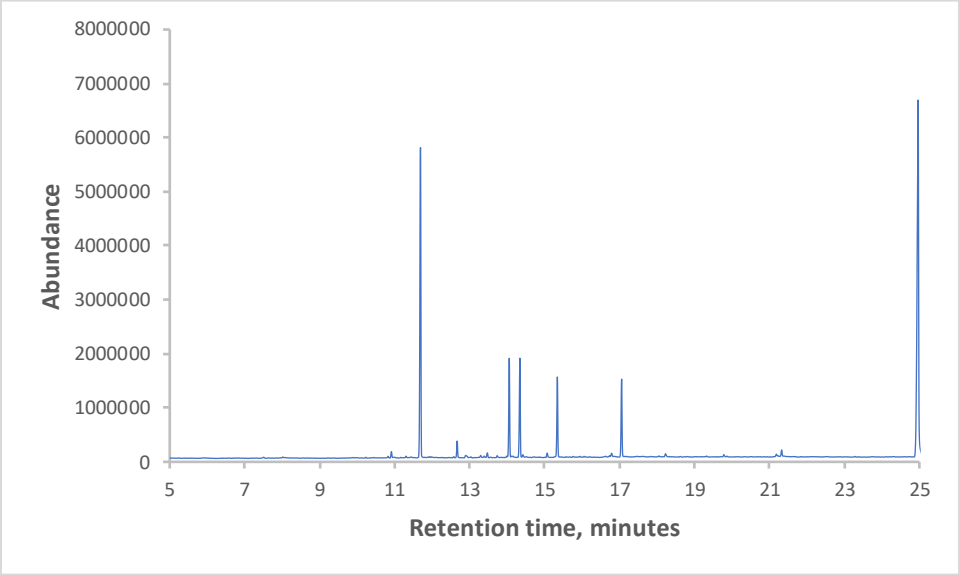


Figure 24 GC-MS chromatographic profile of the sample of pottery that in contact with red wine for 3 months in laboratory. Peak identification in Table 9

Table 9 Identified phenolic acids by GC-MS analysis for the samples were in contact with red wine for 3 months in the laboratory

Retention time	**Identification**
10.83	2-Hydroxyglutaric acid, TMS
10.92	Tartaric acid derivative
11.70	Tartaric acid, TMS
12.59	Hydrocinnamic acid derivative
12.68	Vanillic acid, TMS
13.31	Protocatechuic acid, TMS
14.07	Syringic acid, TMS
14.35	p-Coumaric acid, TMS
24.98	Tetratriacontane, internal standard

It is unfortunate to mention that there were no evidences of any of the listed phenolic acids in the selected samples of São Cucufate, which reshapes our hypothesis of the use of these amphorae as wine container. It drives us to think that possibly these amphorae were used and/or reused for the purposes of storing or transporting plant remains, vegetal oil, animal and aquatic products.

Moreover, it's also possible that the primary purposes of the amphorae was to transport wine and re-uses after primary usage can yield the chemical compounds associated with the secondary, tertiary etc. uses

of the amphorae. For the better understanding of the uses of these amphorae sherds it is essential to conduct further research with the combination of much more sophisticated archaeometric approaches.

4.2 Analysis of lead profile in amphorae by LA-ICP-MS

Laser Ablation - Inductively Coupled Plasma - Mass Spectrometry (LA-ICP-MS) analytical technique is one of the most important mass spectrometric multi-elemental analytical techniques for the characterization of solid samples in materials science and it is characterized by an accuracy of the determinations as good as that obtained with neutron activation analysis (NAA), commonly used in the past for provenance study of ceramics (Niziolek, 2013). LA-ICP-MS is a highly sensitive microprobe technology with many potentials in chemistry based archaeological research. ICP-MS is a versatile technique that enables determination of a wide range of elements with ultra-low detection limits and can easily be coupled to sample introduction systems, allowing in situ analysis of solids (Neff, 2012). Laser sampling offers many advantages, including application to both electrically conducting and non-conducting samples with minimal physical sample preparation. By controlling wavelength and pulse length of the laser, it is possible to remove micro samples from a surface at different levels of depth (Speakman et al., 2002).

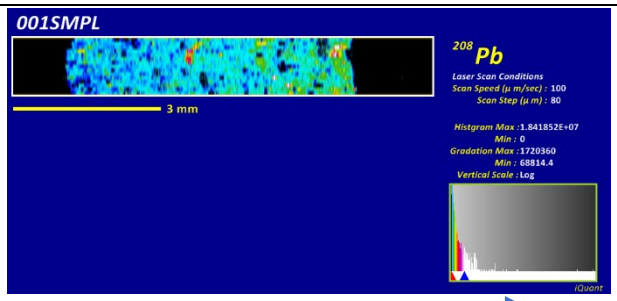
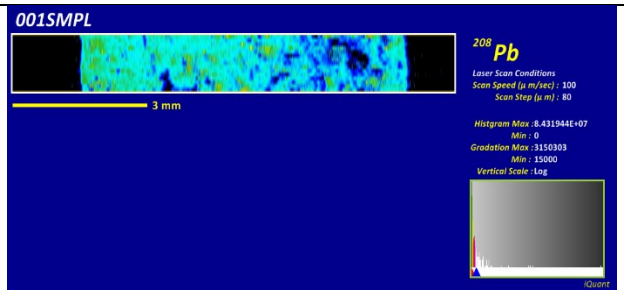
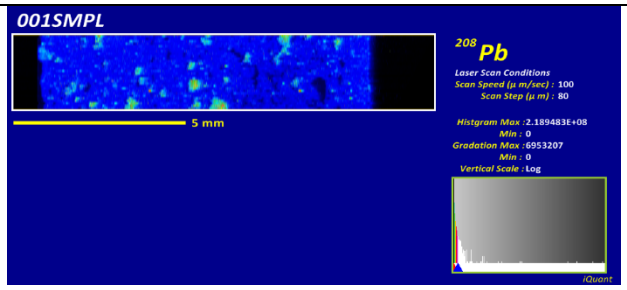
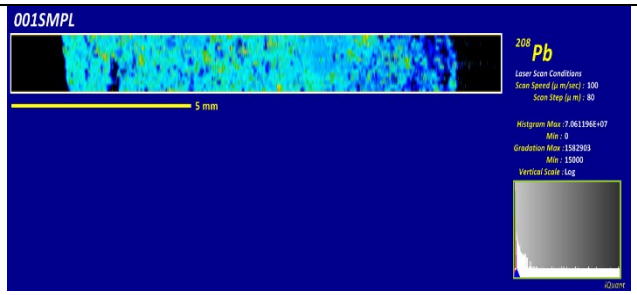
According to Pliny and Columella, Romans boiled grape must in lead vessels to concentrate sugars and allow lead to sweeten wine (Reddy & Braun, 2010). So, lead (Pb) should be more concentrated on the internal part of the amphorae sherds which might have been in contact with lead enriched products. At this stage of the study, we tried to correlate Lusitana 9 / Sado 2 amphorae sherds which showed a chemical fingerprint of resinous materials (*Pinaceae sp.*), with the lead content in the depth profile of samples, which could justify the hypothesis of this research. Wine biomarkers presence was not taken into account, as no specific wine biomarker was detected in the studied samples.

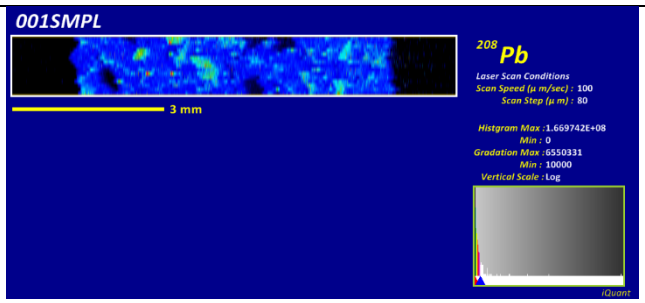
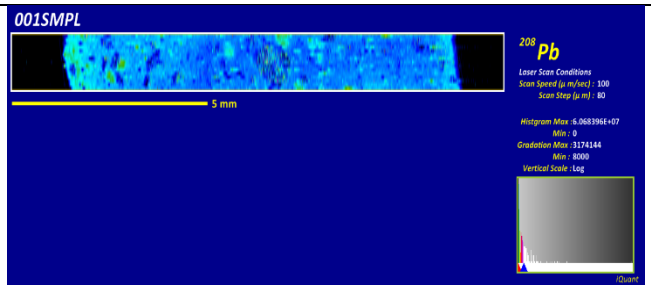
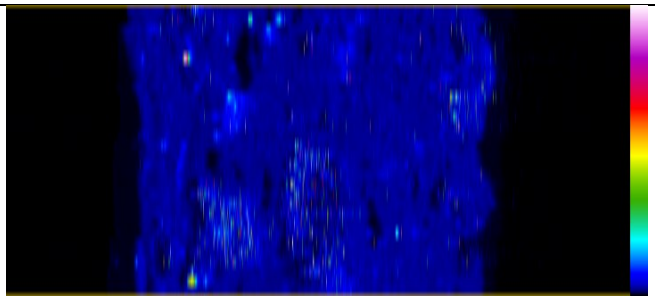
For this purpose, LA-ICP-MS technique was applied for checking if the samples (12 amphorae sherds of São Cucufate) were in contact with this type of lead-enriched wine. This can be done by observing the lead enrichment in depth profile of samples using LA-ICP-MS. Based on depth profile mapping, we could group the amphorae sherds into homogenous lead (Pb) contents and heterogenous lead (Pb) contents.

Homogenous lead (Pb) contents

Some amphorae sherds showed a similar distribution of lead on the whole surface (inside to outside) of depth profile. For these samples, it is possible that the lead (Pb) could be the result of contamination from the soil in which they were buried.

Table 10 Lead (Pb) mapping of the samples chosen for LA-ICP-MS analysis, showing homogeneous Pb content

Sample	LA-ICP-MS Pb mapping
CEG 79.986	 Inside → Outside
CEG 80.767	 Inside → Outside
CEG 81.967	 Inside → Outside
CEG 81.977	 Inside → Outside

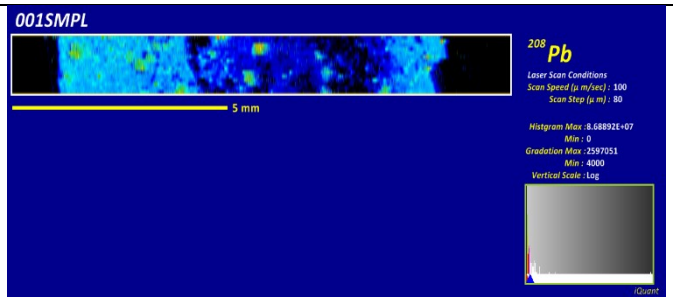
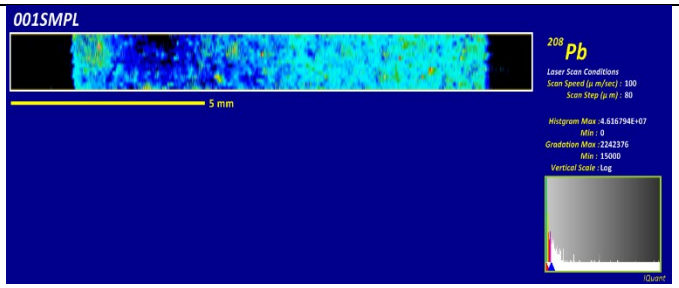
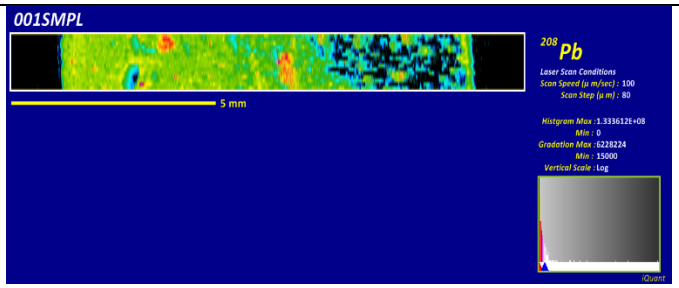
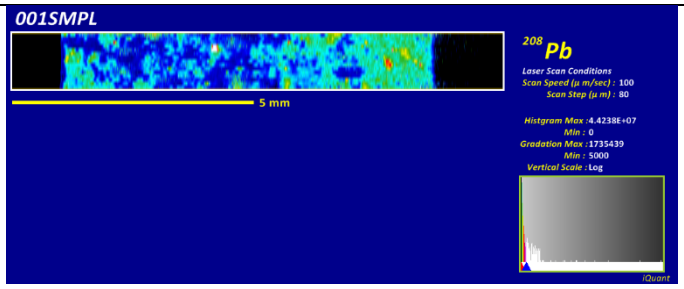
Sample	LA-ICP-MS Pb mapping
CEG 82.5214	 Inside → Outside
CEG 83.3771	 Inside → Outside
CEG 84.5245	 Inside → Outside

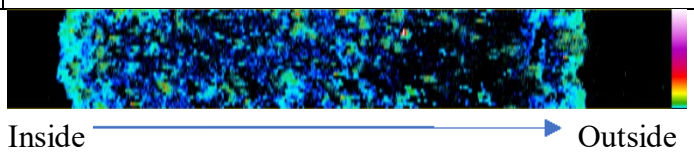
Heterogenous lead (Pb) contents

Among the twelve studied samples, five samples were identified with an heterogenous distribution of lead (Pb) contents. Two samples, CEG 80.711 and CEG 83.2696 exhibited a more intense lead (Pb) distribution on the internal surface. These distributions might have been the result of contact with lead enriched wine products. From the organic residue analysis, these two samples also yielded diterpenic compounds, which can be associated with the resins that exude from plants of the *Pinaceae* family. If we had identified the combination of wine phenolic acids in these two samples as well, then it would be easier to reach the consideration that these amphorae were used for storing or transporting wine which was enriched in lead. Even though it was not possible to trace wine phenolic acids by GC-MS, it's still possible that primarily these amphorae were in contact with lead enriched wine and then went through

several re-uses, enabling to trace only chemical compounds associated with the more recent use of amphorae.

Table 11 Lead (Pb) mapping of the samples chosen for LA-ICP-MS analysis, showing heterogeneous Pb content

Sample	LA-ICP-MS Pb mapping
CEG 80.711	 Inside → Outside
CEG 81.1536	 Inside → Outside
CEG 83.2696	 Inside → Outside
CEG 84.5483	 Inside → Outside

Sample	LA-ICP-MS Pb mapping
CEG 83.3793	

The samples CEG 81.1536 and CEG 84.5483 exhibited a more intense distribution of lead on the outer part of the amphorae sherds. This phenomenon can be suggested as a contamination from the soil where they were buried for a long period of time.

CHAPTER 5: FINAL REMARKS

5.1 Implications of research findings

Based on the objectives of this research, GC-MS was used to analyze organic residues and wine biomarkers from the twenty selected samples and LA-ICP-MS was applied to check the presence of the lead contents in the depth profile of twelve selected samples. GC-MS enabled to identify a wide range of chemical compounds.

The diterpenoid acids, such as pimaric, isopimaric and abietic acids are known to be present in pine resin were identified in some of the samples. The presence of diterpenoids can be an evidence that the studied amphorae were waterproofed with resinous substances for carrying liquid or semi liquid products. It is unfortunate that no evidences of phenolic acids were found with wine biomarker extraction and analysis by GC-MS. It was not possible to make a direct correlation between wine biomarkers, presence of resinous substances and lead (Pb) depth profile.

In the organic residue analysis biomarkers of a plant oil together with *Pinaceae* sp. pitch was identified, which is known to have been used to impermeabilize the inner surface of wine and fish sauce amphorae. Due to the lack of references from the classical authors, the impermeabilization process of oil amphorae is obscure among the historians and archaeologists (Romanus et al., 2009). The treatment of oil *dolia* with amurca and plant gums is a well-established concept instead (Forster et al., 1955). Still, it's quite possible that wine amphorae could be reused for carrying oil or fish products or vice-versa.

It is already mentioned that slightly more polar compounds, such as unsaturated fatty acids and products of their degradation (diacids and hydroxyacids) are very vulnerable to complete deterioration. More polar organic compounds are usually likely to interact strongly with the structural components of ceramic. Nevertheless, the presence of wide range chemical compounds is a clear identification of use and reuses of amphorae possibly for plant products, vegetal oil, aquatic and animal products during its lifetime.

5.2 Research contribution and recommendation

The findings of this research will rebound to the benefit of archaeometric research considering that organic residue analysis plays significant role for revealing past human diet, agriculture, socio-economic structure, trade-transport and cultural diversity. The adopted protocols for total lipid and wine biomarker extraction were previously applied for different research projects, in the field of Heritage Sciences.

Although it was not possible to identify wine biomarkers in the amphorae sherds (which was one of the main prospects of this research), several chemical compounds from total lipid extraction allowed to determine that these amphorae were in contact with plants and animals' products, which is necessarily a clear indication of use and reuse of the amphorae during their lifetime. LA-ICP-MS analysis is a sophisticated analytical technique with a wide range of applicability in archaeometric research, which was also applied and found appropriate for checking the lead (Pb) contents in the depth profile of amphorae sherds.

The results derived from the recommended approaches will contribute in future research to approximate related outcomes, which can be an exposition for the necessary modification, change or alternation of methodological approaches. Thus, a more improved and result oriented approach can be developed which will uncover critical areas in this field of study.

5.3 Limitations

Every scientific research is associated with lot of limitations from the beginning to the end and it is essential to minimize the limitations for a better outcome. This research was not an exception in terms of limitations. Both analytical methods GC-MS and LA-ICP-MS are destructive and require a complex

and lengthy process of sample preparation by several steps (drilling, cleaning, breaking, cutting, grinding, mounting in epoxy resin, polishing).

It was found that almost all the samples (amphorae sherds from São Cucufate) went through several phases of contamination by handling, washing, storing, plastic wrapping and overall documentation. In all samples, several chemical compounds related with plastic contamination were identified.

For the absence of wine biomarkers in the samples which yielded resinous substances by GC-MS and heterogenous distribution of lead (Pb) content in the depth profile by LA-ICP-MS, it was not possible to correlate resin, lead and wine all together which could justify the hypothesis of Lusitana 9 / Sado 2 amphorae as wine container.

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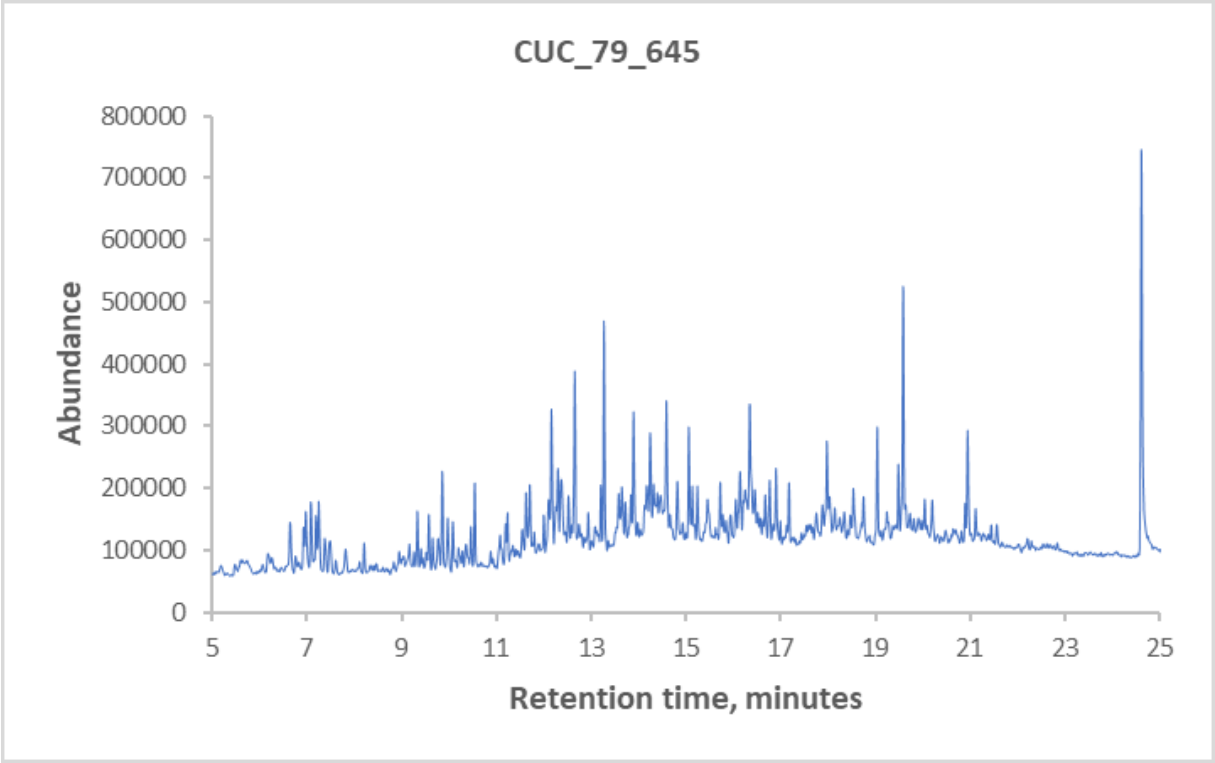
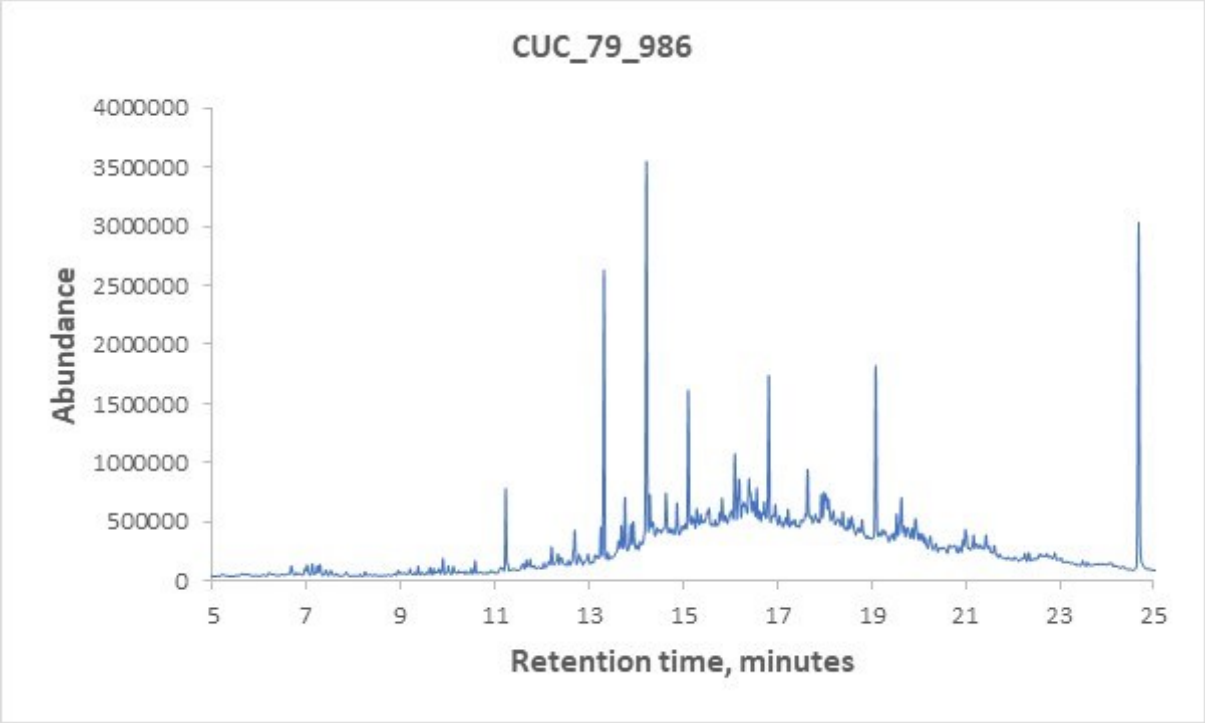
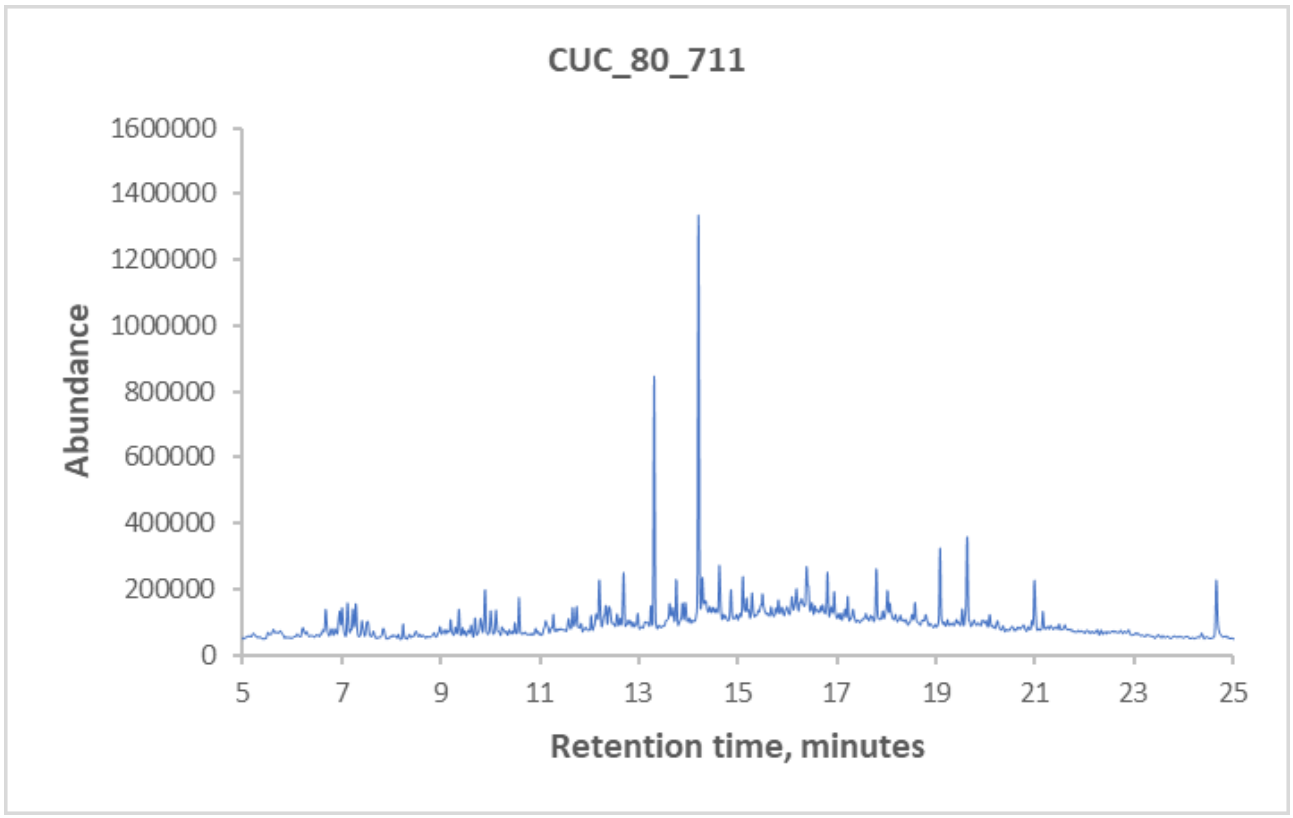
Appendix I

GC/MS chromatogram of samples (IS = internal standard, n-tetratriacontane)



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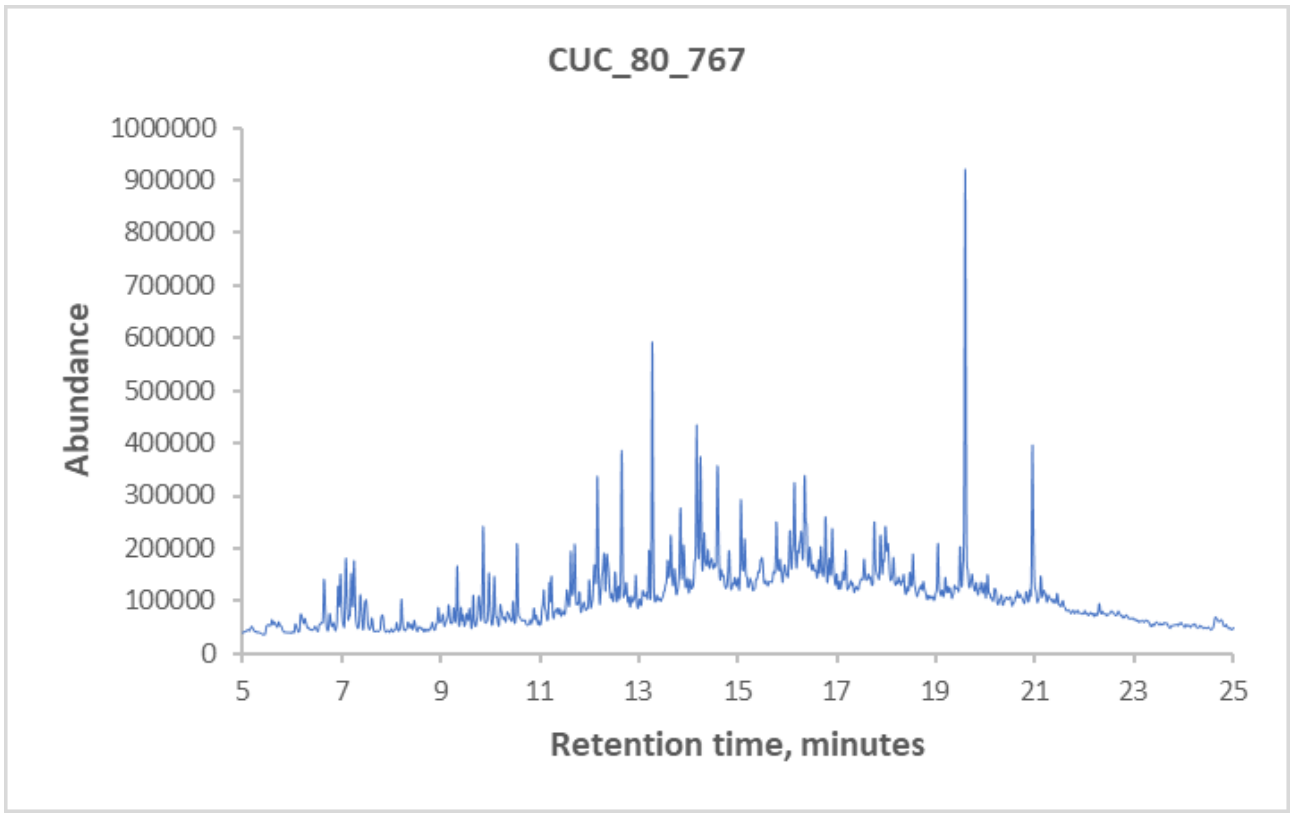
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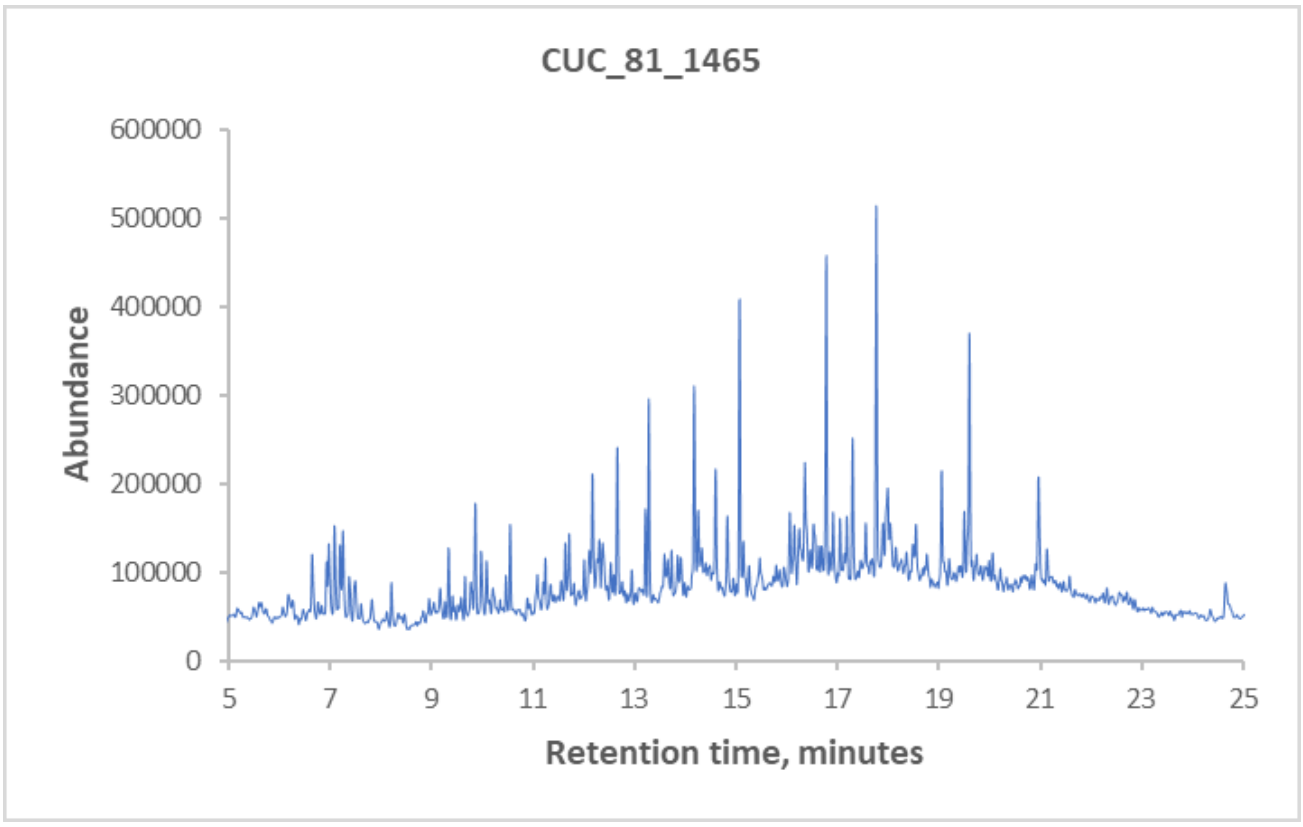
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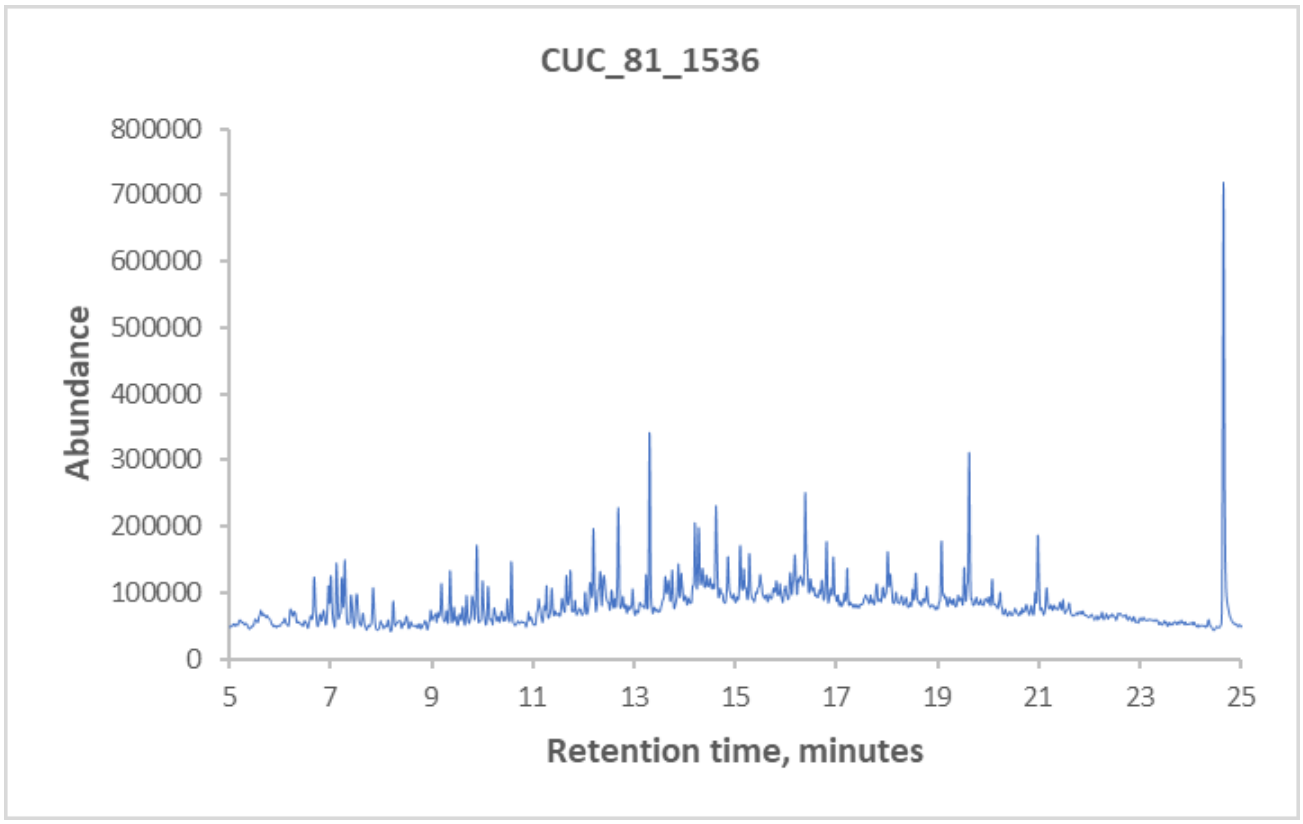
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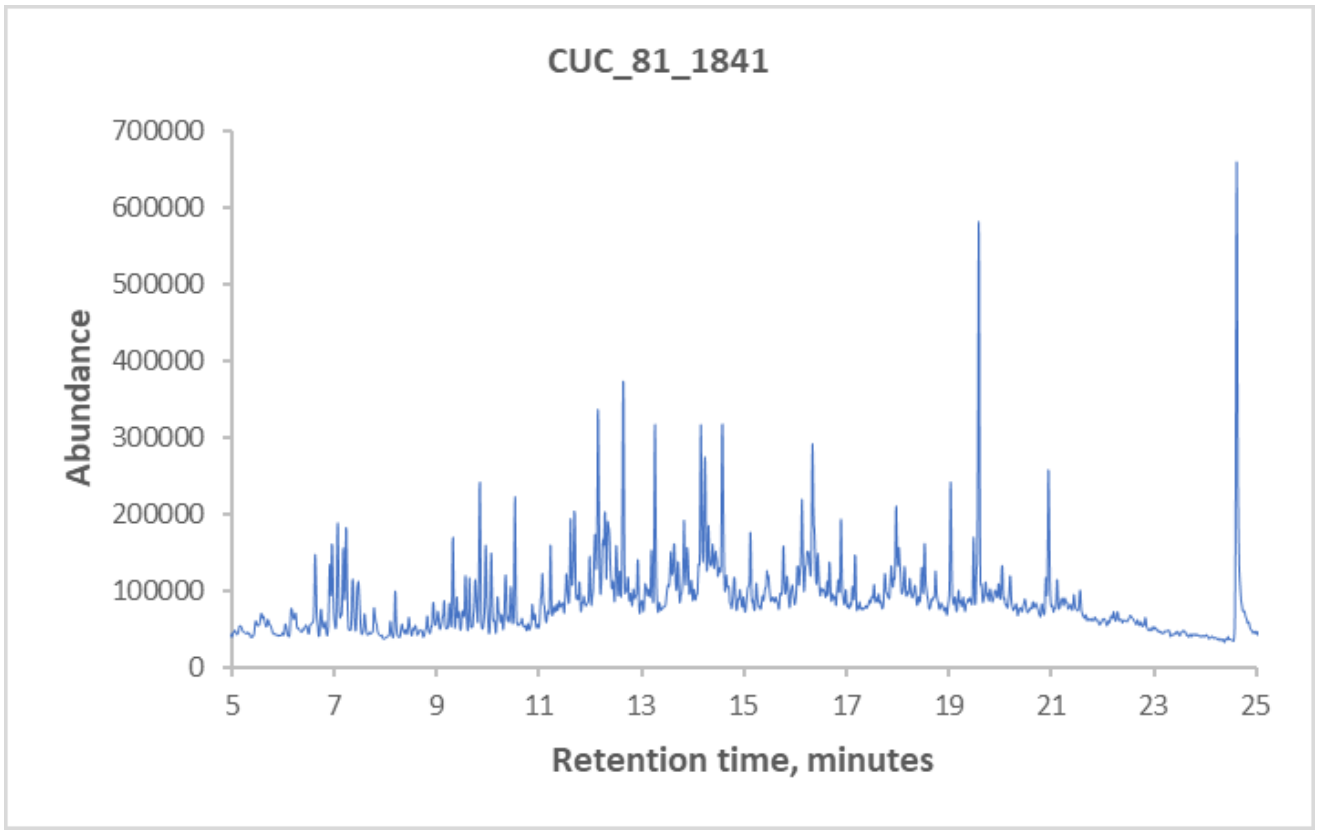
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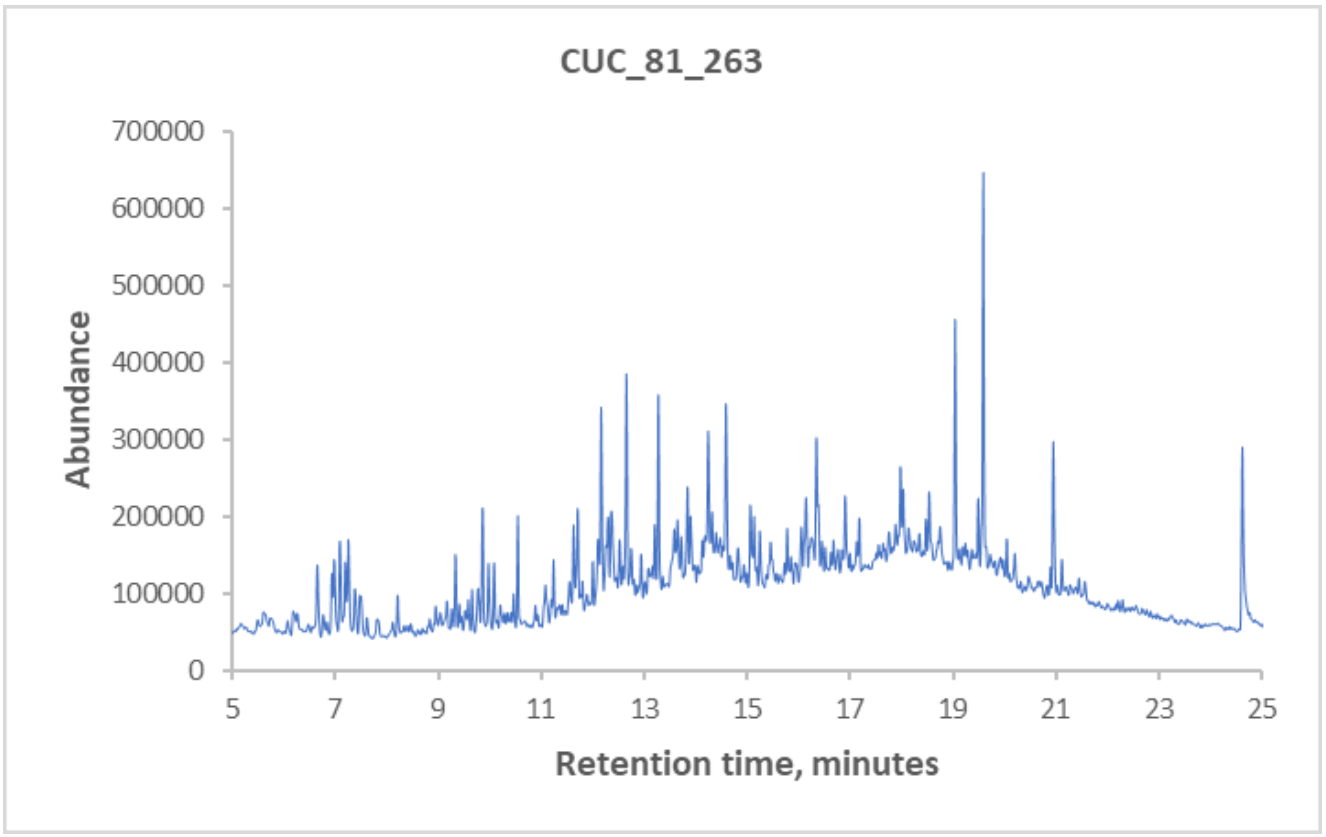
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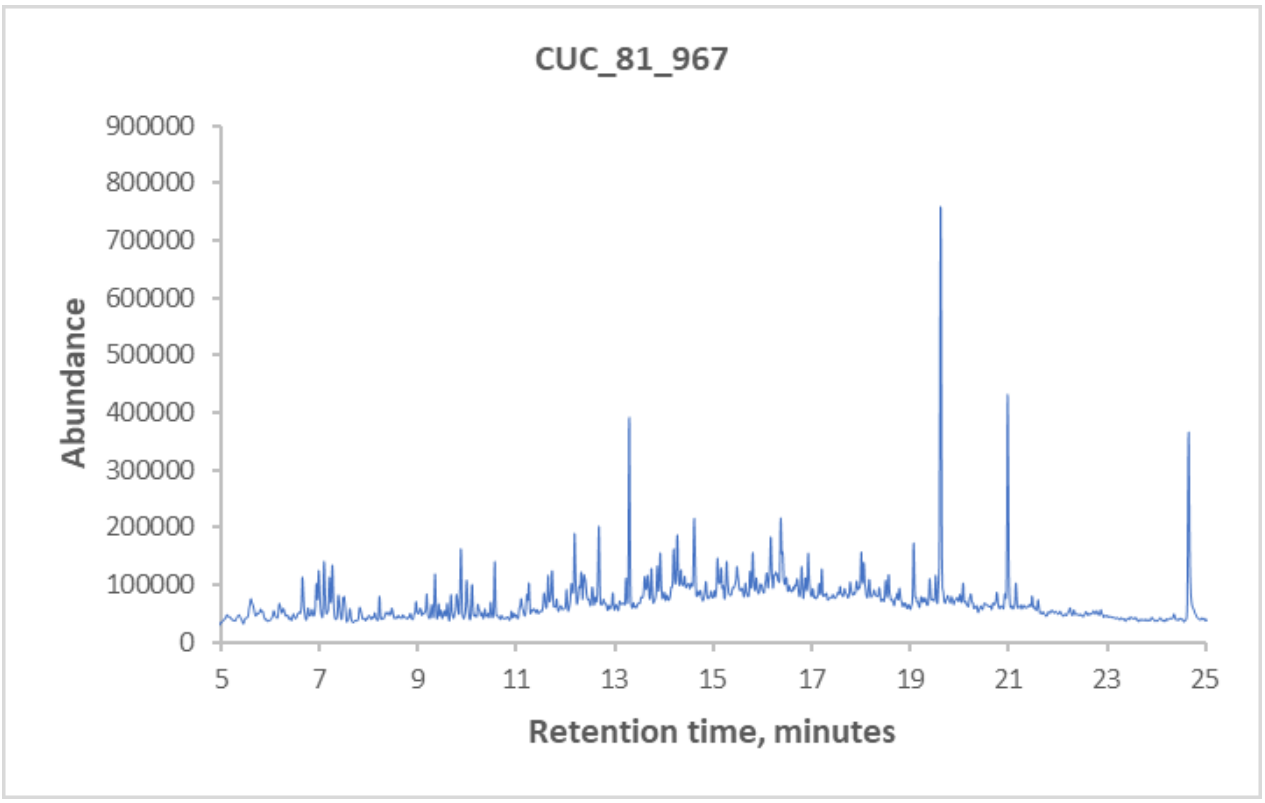
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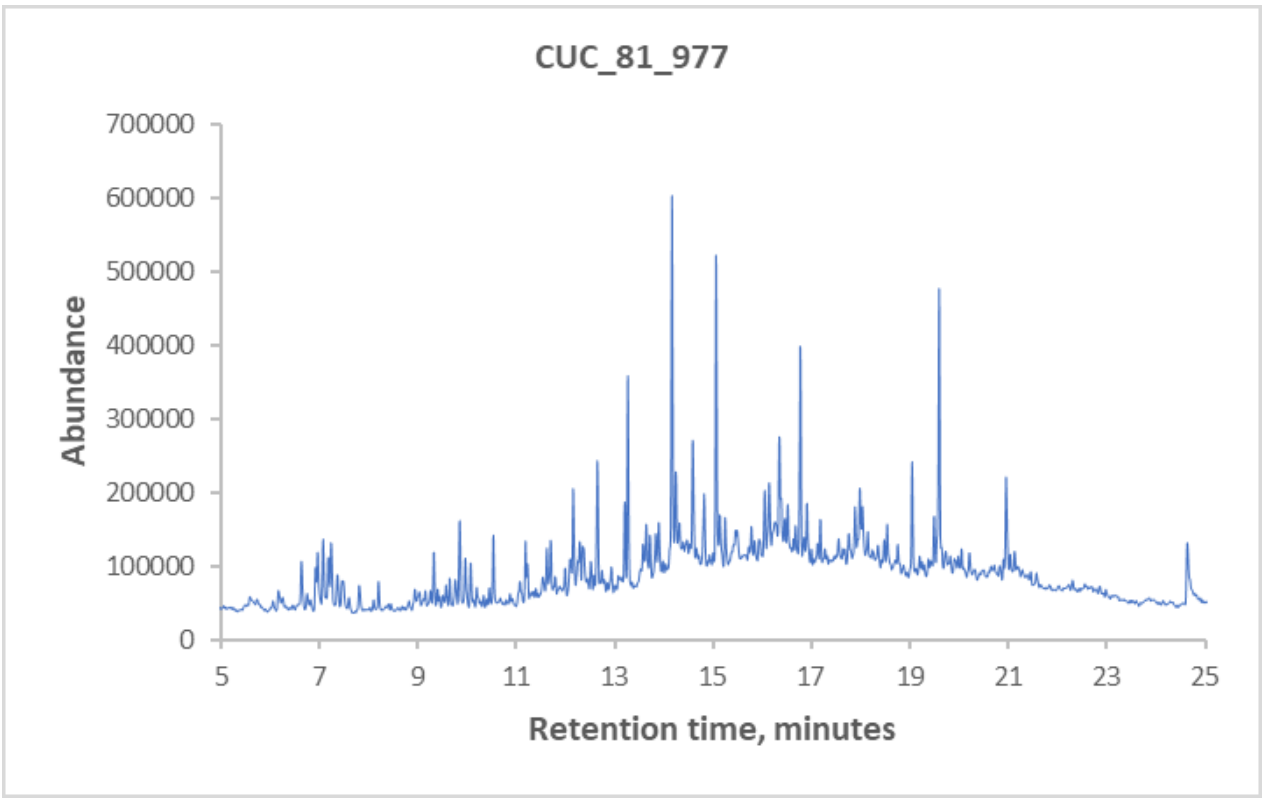
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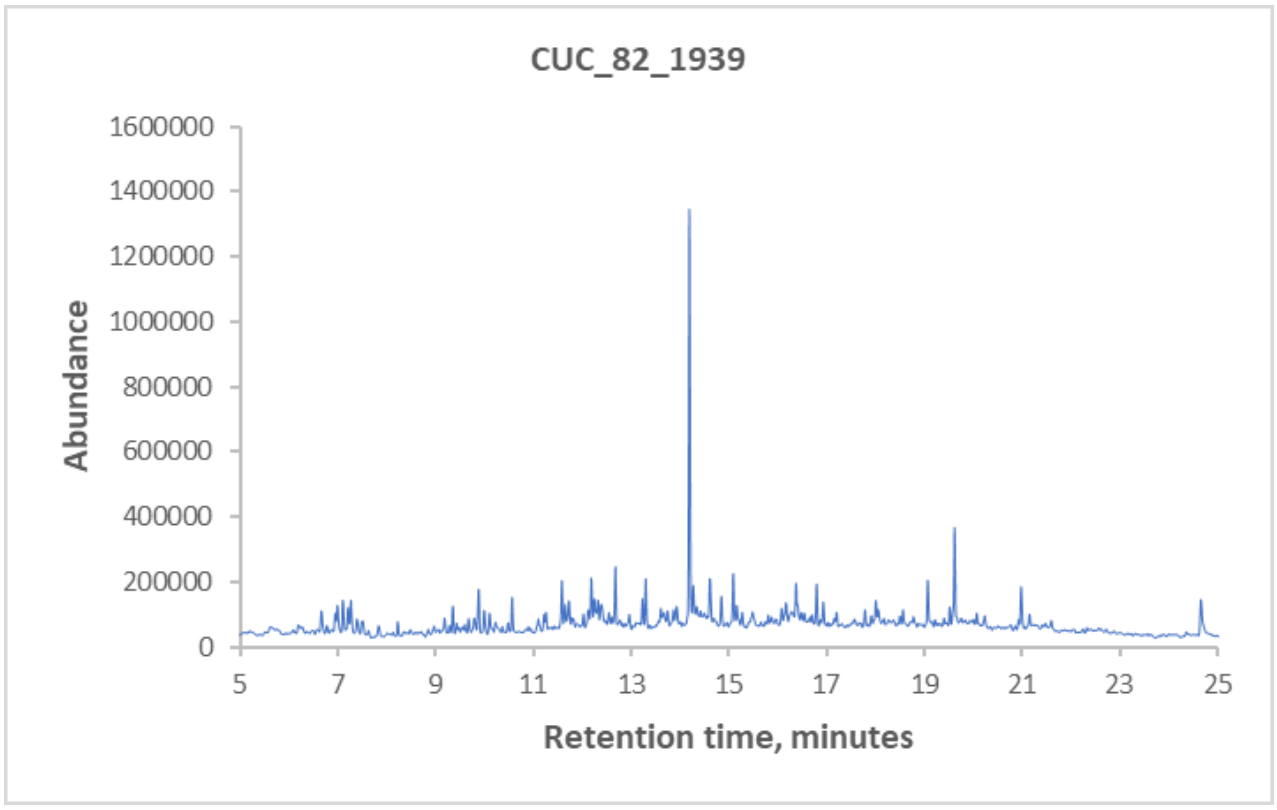
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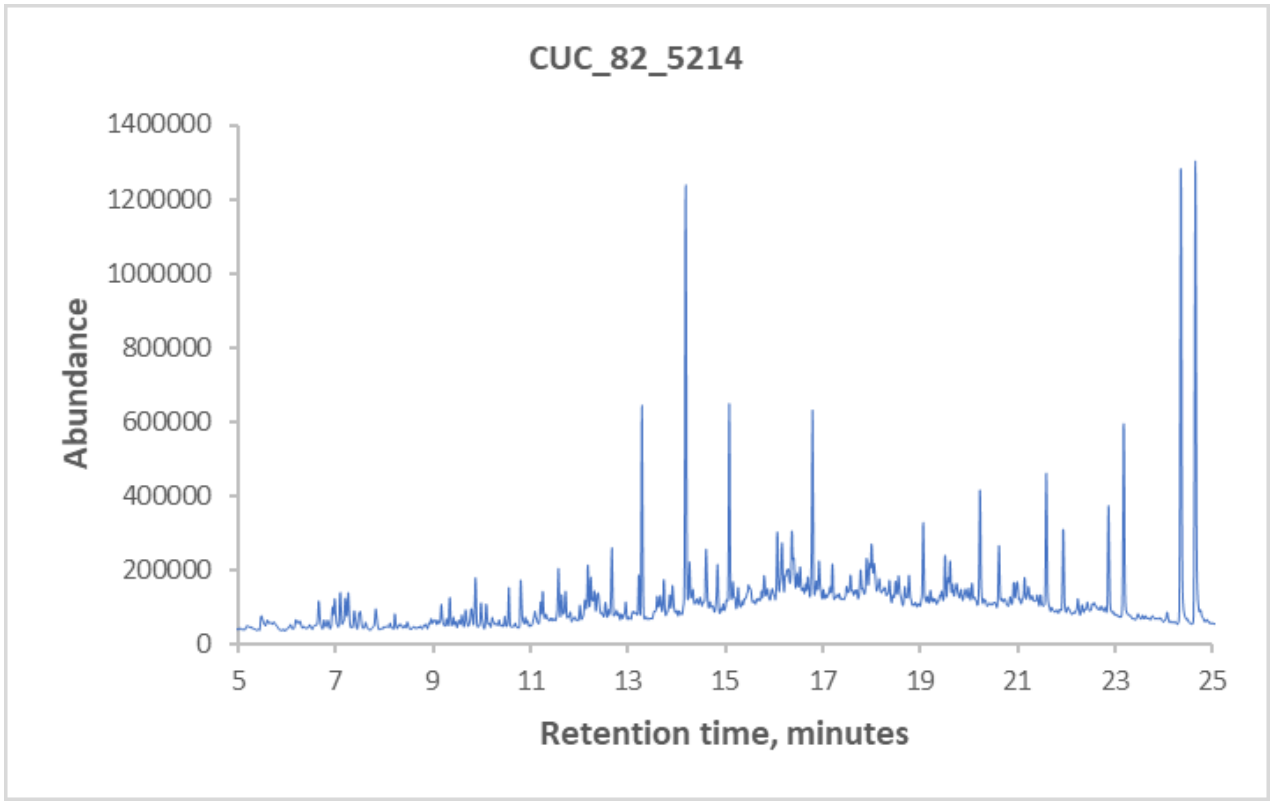
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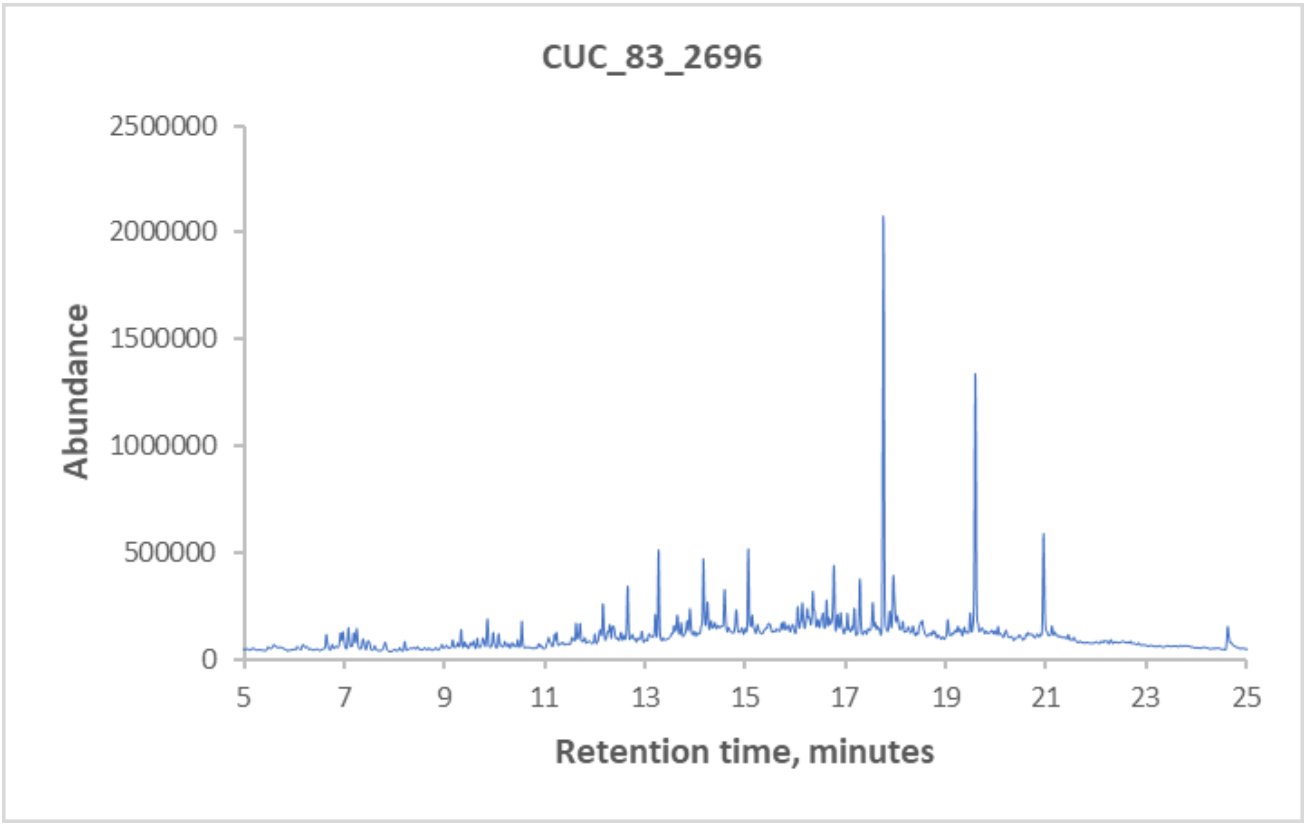
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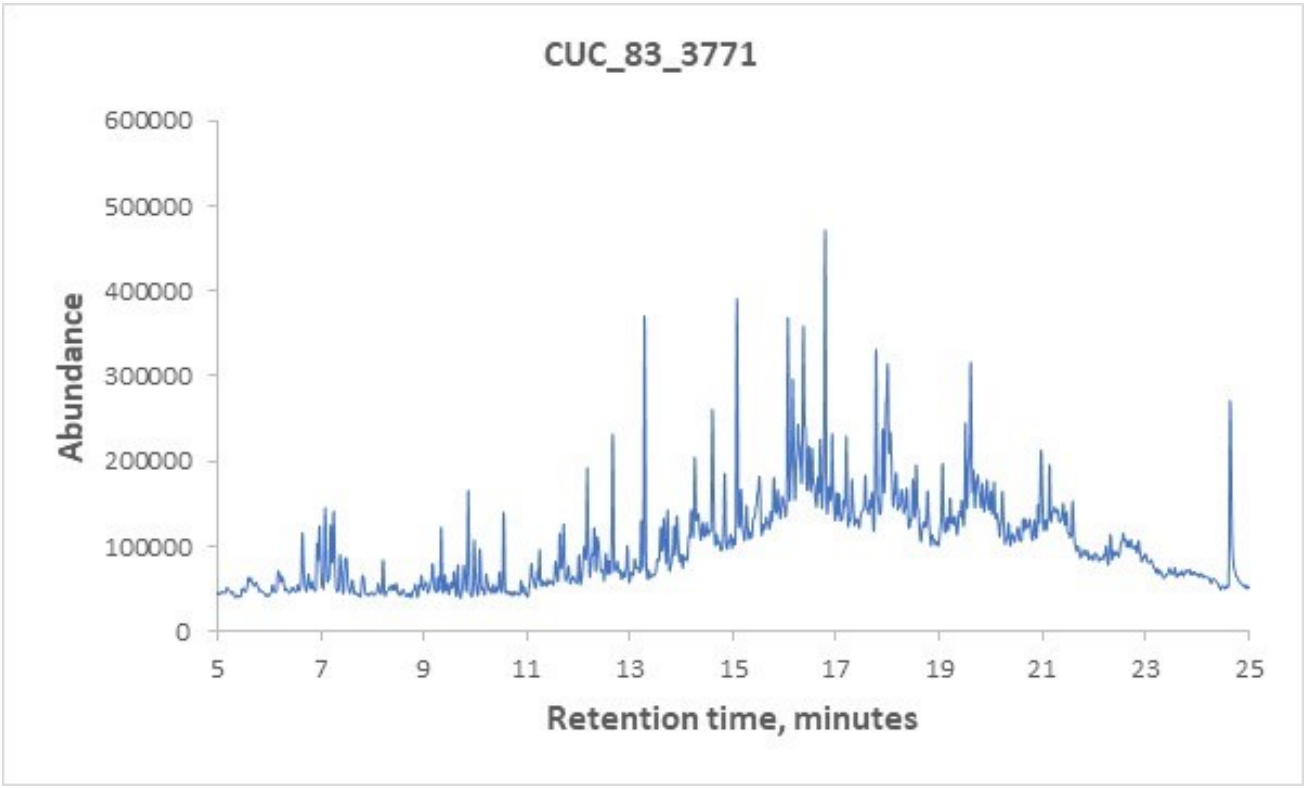
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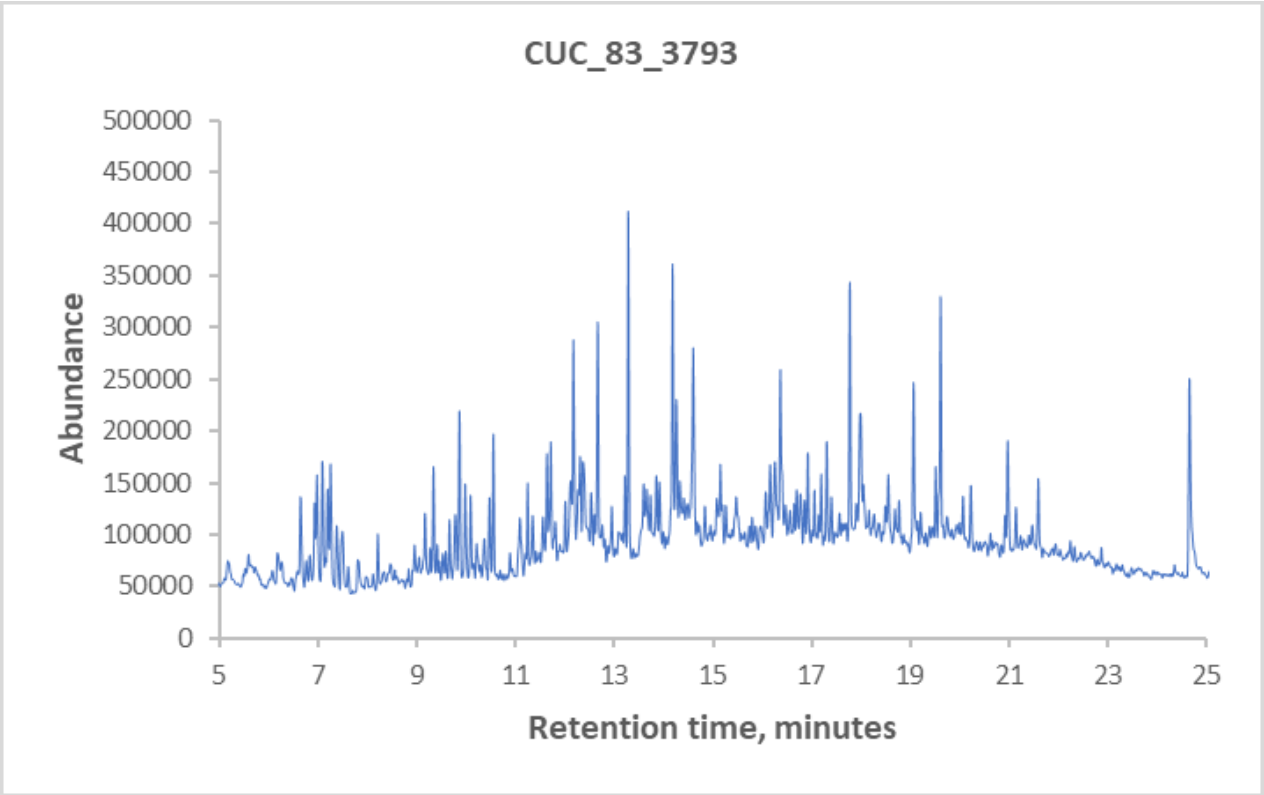
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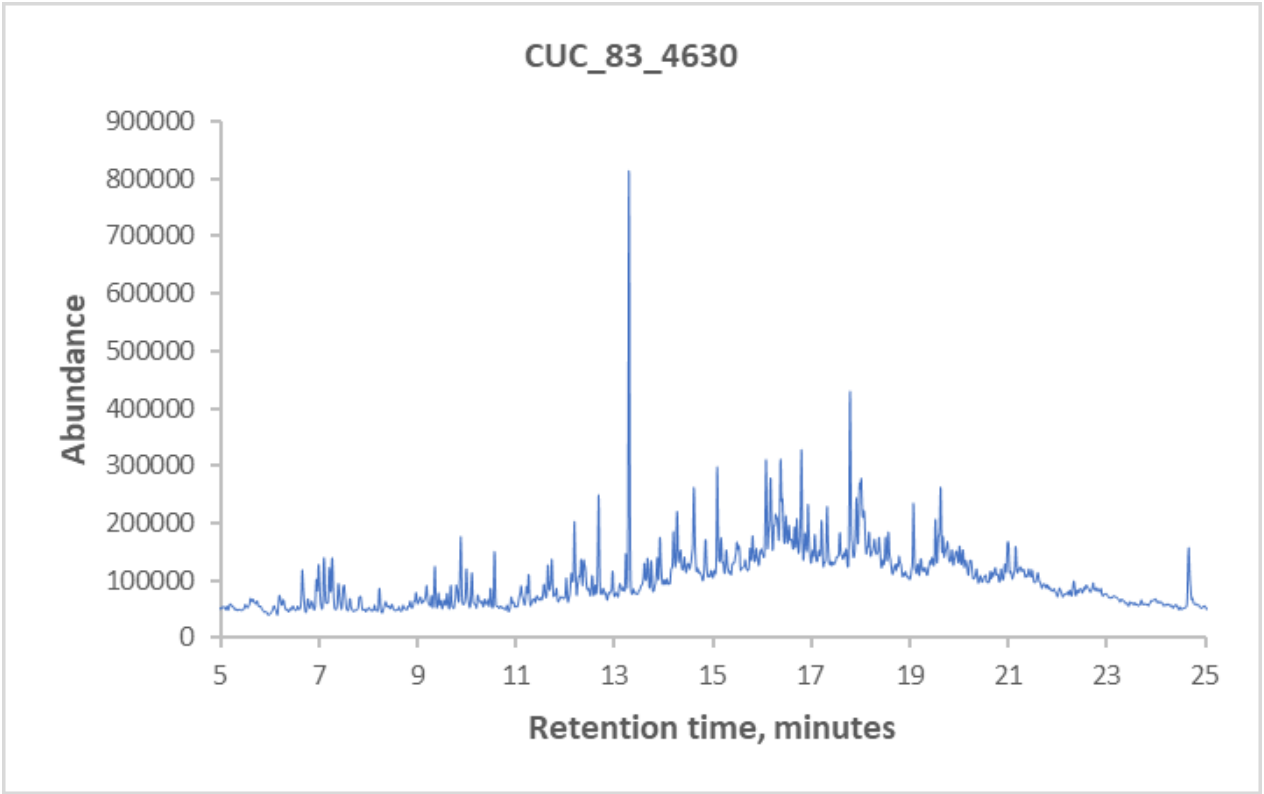
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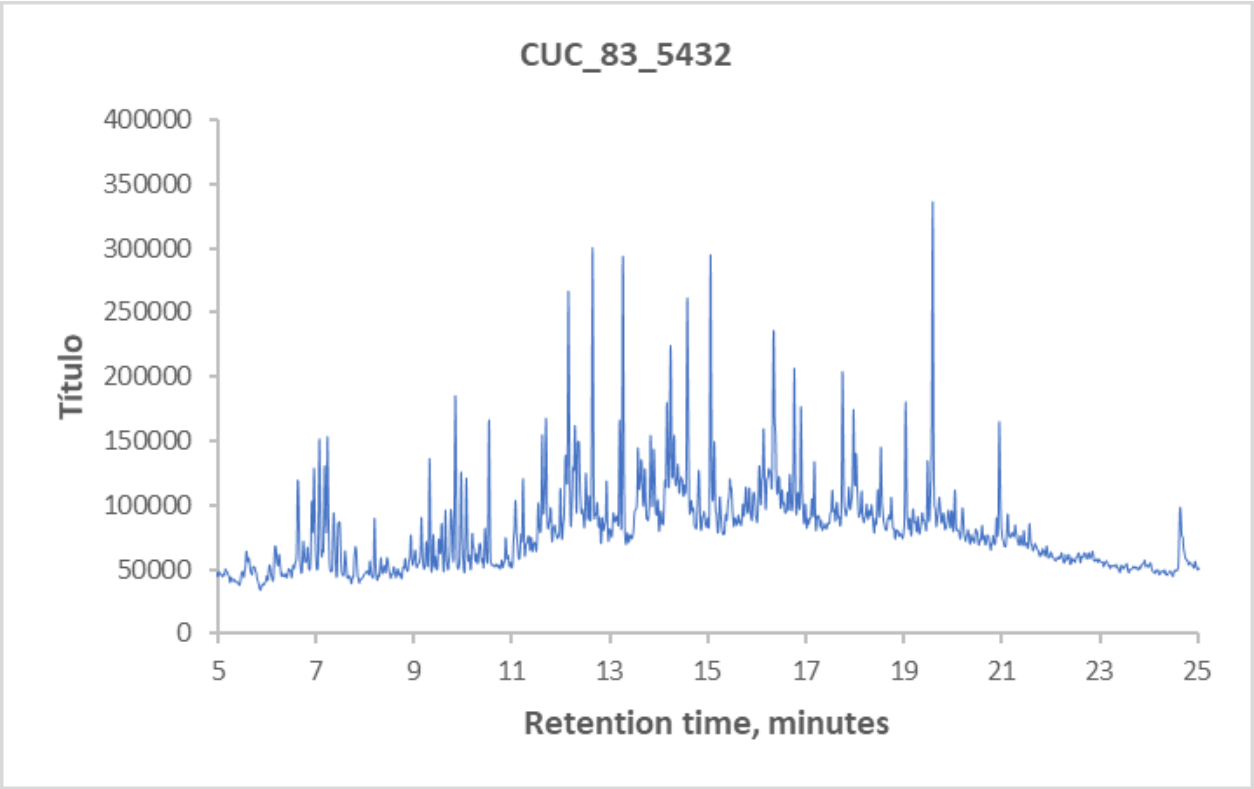
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300000
250000
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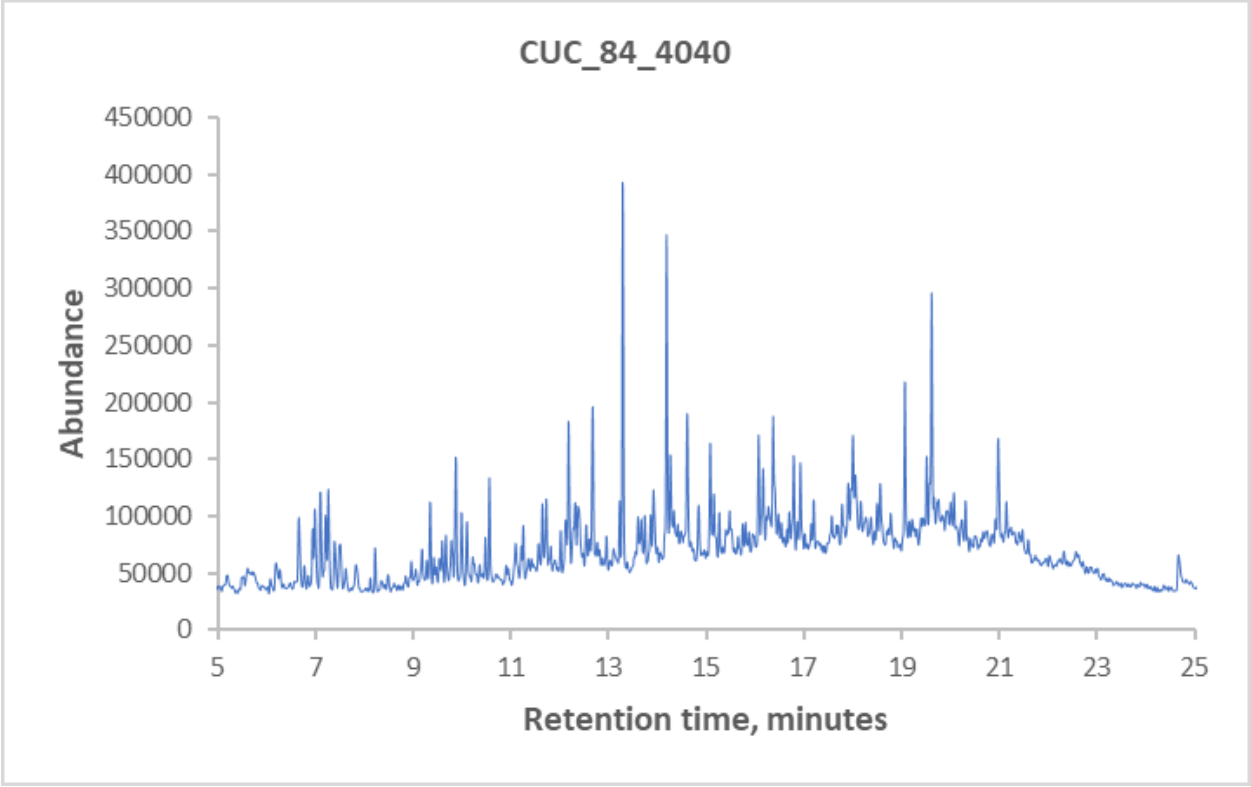
7
9
11
13
15
17
19
21
23
25
Retention time, minutes


CUC_84_4040
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350000
300000
250000
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100000
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7
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11
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23
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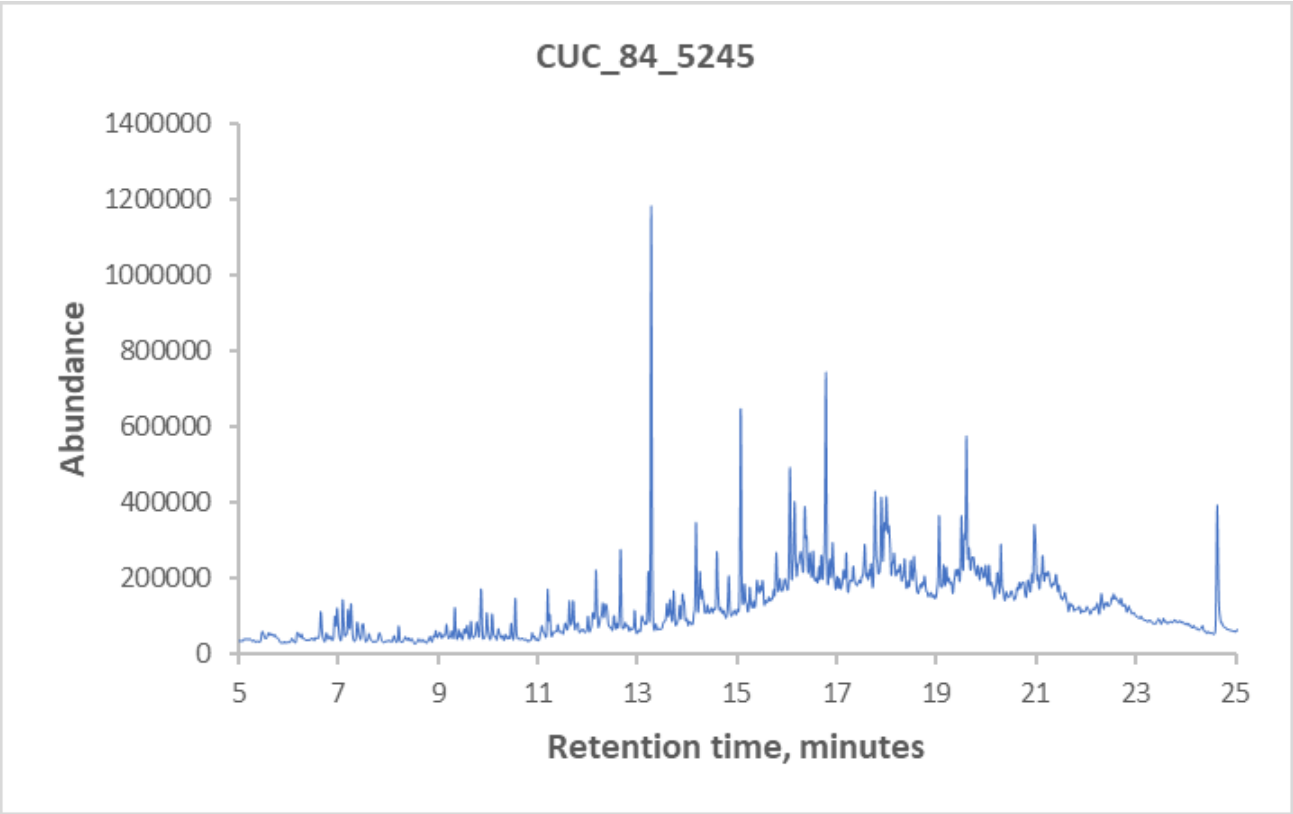
Retention time, minutes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Abundance
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1200000
1000000
800000
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200000
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19
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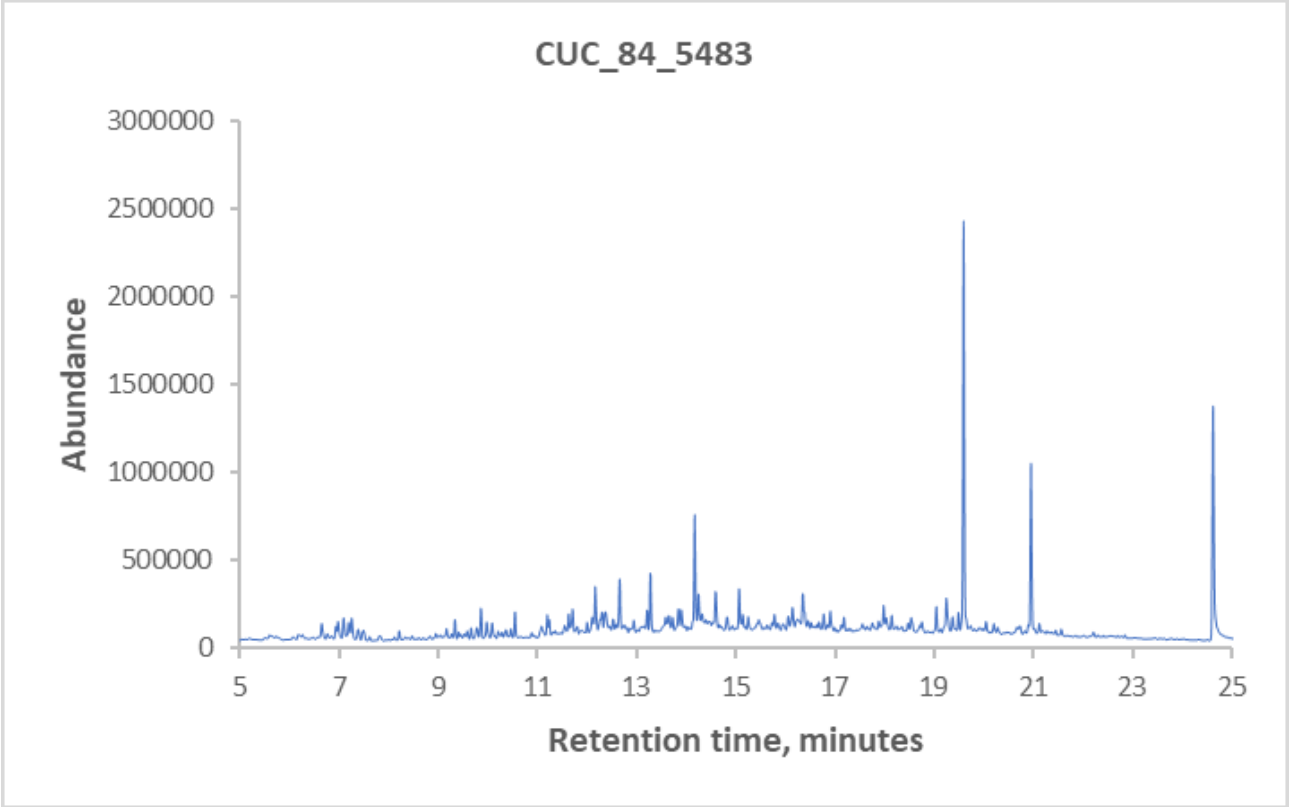
23
25
Retention time, minutes


CUC_84_5483
Abundance
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1500000
1000000
500000
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21
23
25
Retention time, minutes